

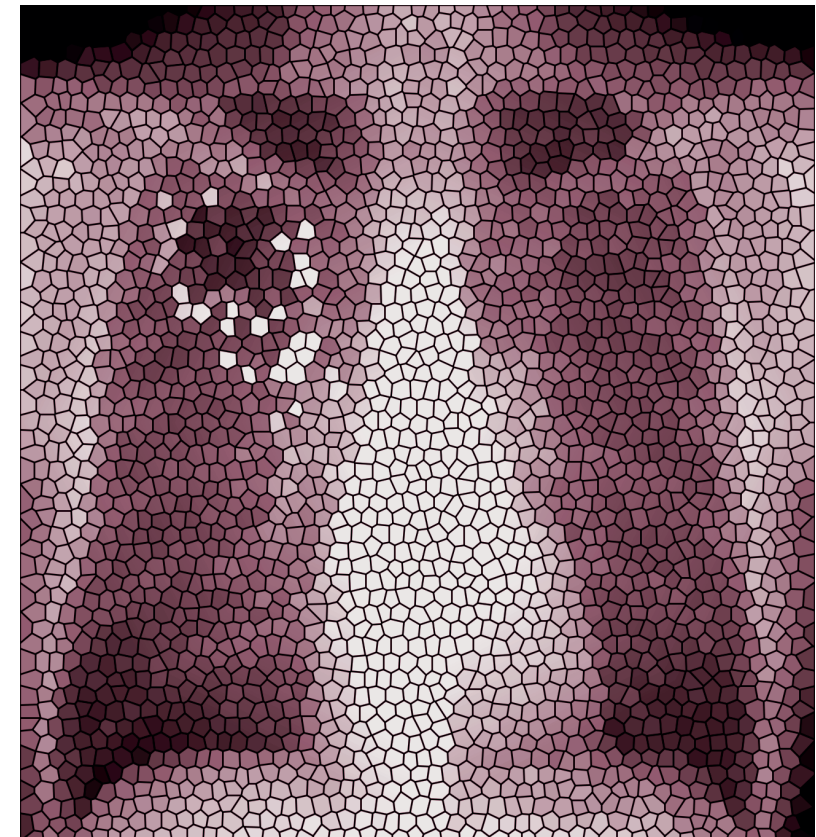
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Novel treatment strategies for multidrug-resistant tuberculosis

Lina Davies Forsman

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Institutet**



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NOVEL TREATMENT STRATEGIES OF MULTIDRUG-RESISTANT TUBERCULOSIS

Lina Davies Forsman



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Novel treatment strategies for multidrug-resistant tuberculosis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Public defence at Karolinska Institutet on December 7, 2018 at 09.00

Welandersalen, Entrance B2, Floor 00 (B2:00), Karolinska University Hospital Solna

To all staff and patients at the Church of Scotland Hospital, Tugela Ferry, Kwa-Zulu Natal,
South Africa

'And death shall have no dominion'

Dylan Thomas 1933

ABSTRACT

Tuberculosis is the leading cause of death from a single infectious agent in the world, surpassing the annual death toll of both malaria and HIV combined. The rise in multidrug-resistant tuberculosis (MDR-TB) has further exacerbated the situation, as this form of TB is more difficult and time-consuming to treat, with dismal cure rates. There is an urgent need to improve treatment of MDR-TB and to avoid the development of resistance during TB treatment. This thesis will delve deeper into the intricate relationship of bacterial resistance, drug exposure and host response, with the aim of exploring the role of repurposed drugs, the importance of the level of drug resistance of TB-drugs as well as therapeutic drug monitoring in the fight against TB and MDR-TB.

Repurposing already approved drugs may accelerate the availability of new drug alternatives and two such alternatives were explored in **Study I & II**. The minimum inhibitory concentration (MIC) *in vitro* was determined for trimethoprim-sulfamethoxazole and meropenem-clavulanic acid, showing good activity against highly drug-resistant *Mycobacterium tuberculosis* (*M.tuberculosis*) isolates. Explorative pharmacometric modelling showed a high probability of target attainment for clinically achievable doses of trimethoprim-sulfamethoxazole (800 mg sulfamethoxazole thrice daily). However, there are no clinical trials evaluating the effect of trimethoprim-sulfamethoxazole in MDR-TB treatment. Meropenem-clavulanic acid is recommended by World Health Organization as an add-on agent in difficult to treat cases.

The importance of the level of bacterial resistance was explored in **Study III**, a national cohort study in Sweden including all MDR-TB cases from 1992-2014 (no= 158). Increments of MIC for fluoroquinolones, rather than binary resistance testing, were associated with increased risk of unsuccessful treatment outcome. A similar association was seen for increasing age and patients with diabetes. Furthermore, pyrazinamide treatment was associated with reduced time to sputum culture conversion for patients with pyrazinamide-susceptible *M.tuberculosis* isolates.

Bacterial resistance, as well as individual drug exposure, were studied in **Study IV**, where drug concentrations were measured in a prospective cohort study of susceptible TB. The drug exposure of first-line drugs in TB-patients was often lower than recommended (16-42%), despite the use of recommended dosages. The number of patients with low drug concentrations of rifampicin was particularly pronounced (13/31, 42%), with great inter-individual variability. When taking individual MICs into account, the ratios of drug exposure and the MICs were still low, possibly contributing to the overall successful treatment outcome. Subtherapeutic drug exposure can be revealed by therapeutic drug monitoring. The result of this study has led to the development of a prospective cohort study in China, studying drug exposure in relation to bacterial MIC in MDR-TB patients.

In conclusion, a more holistic approach, taking the level of bacterial susceptibility, individual drug exposure as well as comorbidities into account, is needed for an individualised, improved treatment of TB. For clinicians, therapeutic drug monitoring might be a useful tool for selected patients, with the aim of preventing acquired drug resistance and improving treatment outcome of TB and MDR-TB.

POPULÄRVETENSKAPLIG SAMMANFATTNING

”Hur kan det få vara så hemskt, frågade jag, hur kan det få vara så hemskt att en del måste dö, när dom inte har fyllt tio år ens?”

Skorpan, döende i tuberkulos, till Jonathan ur Bröderna Lejonhjärta 1973 av Astrid Lindgren

Från att ha varit en farsot i det svenska samhället under många århundraden, har tuberkulos blivit en ovanlig dödsorsak i Sverige. Så ser det dessvärre inte ut i resten av världen. Faktum är att tuberkulos är den infektionssjukdom som skördar flest liv årligen, mer än HIV/AIDS och malaria sammanlagt. Förra året dog mer än en och en halv miljon människor av tuberkulos. Tuberkulos är en social sjukdom som starkt påverkas av den miljö man lever i och där trångboddhet, undernäring och bristande tillgång till sjukvård ökar risken för insjuknande. Tuberkulos orsakas av bakterien *Mycobacterium tuberculosis* (*M.tuberculosis*) och sprids via luften i små droppar via hosta från en tuberkulos-sjuk och kan vid sjukdom ge symtom som långvarig, ibland blodig hosta och utmärgling. Under framförallt 1700-talet till 1900-talets första hälft var Sverige liksom resten av Europa svårt drabbat av tuberkulos, eller lungsot som det också kallades. Så här beskrev stadsläkaren och professorn Klas Linroth tuberkulosens framfart i Stockholm 1880: *”Lungsoten, vår stads liksom andra orters värsta mordengel”*. Under början av 1940 var antal nya fall per år över 300 per 100 000 innevånare, liknande situationen i Sydafrika i dag.

Tuberkulos är möjlig att utrota då människan är enda smittbäraren, så varför dog så många människor förra året av en behandlingsbar sjukdom? Bristande tillgång till sjukvård, läkemedel, utebliven diagnostik och smittspårning samt ökad andel av läkemedelsresistent, dvs. motståndskraftig mot ett eller flera läkemedel, tuberkulos är några delförklaringar. Känslig tuberkulos, utan läkemedelsresistens, behandlas i minst 6 månader med fyra läkemedel. Multiresistent tuberkulos (MDR-tbc) definieras som *M.tuberculosis* med resistens mot de två viktigaste läkemedlen rifampicin och isoniazid och med fler än 500 000 nya fall årligen är det ett stort hot mot folkhälsan i världen. Behandlingen av MDR-tbc är upp till två år med många biverkningar och den globala utläkningen är endast 54 %. De enda nya läkemedel som framtagits under de senaste 40 åren är bedaquiline och delamanid. Dessa kan komma att förbättra överlevnaden hos individer med resistent tuberkulos, men bara 10 % av de patienter som behöver dem får tillgång till de nya läkemedlen. Därför behövs billiga, tillgängliga alternativa läkemedel.

Syftet med avhandlingen är att undersöka nya strategier för att förbättra behandlingen av tuberkulos och MDR-tbc genom att:

- återanvända äldre antibiotika och undersöka dess effekt på läkemedelsresistent *M. tuberculosis* (**Studie I & II**)
- studera förhållandet mellan bakteriens grad av resistens och behandlingsutfallet av MDR-tbc (**Studie III**)
- bestämma läkemedelskoncentrationerna i blodet hos tuberkulospatienter i Sverige och förhållandet till bakteriens grad av resistens (**Studie IV**)

Då äldre antibiotika redan finns tillgängliga och ofta är billiga är de en potentiell källa till nya läkemedelsalternativ vid svårbehandlad tuberkulos. Därför studerade vi effekten av Bactrim (trimethoprim-sulfamethoxazole, TMP-SMX), ett sulfa-innehållande läkemedel som tidigare ofta användes mot exempelvis urinvägsinfektioner. Multiresistenta *M.tuberculosis* bakterier på odlingsplattor utsattes för TMP-SMX i stigande koncentrationer för att finna den minsta hämmande koncentrationen, dvs. den lägsta koncentrationen av läkemedlet som dödar all synlig växt (**Studie I**). Vi fann att de koncentrationer som krävdes för att döda bakterierna bör kunna uppnås och tolereras i människa. Vidare undersöktes effekten på multiresistent *M.tuberculosis* av meropenem-clavulansyra (MEM-CLA), ett intravenöst läkemedel som vanligen används vid svåra fall av blodförgiftning (**Studie II**). Här såg vi också god avdödning av tuberkulosebakterien i labbmiljö när MEM-CLA tillsattes. TMP-SMX och MEM-CLA har dock inte utvärderats i tillräcklig grad i kliniska studier för att säkerställa dess effekt hos patienter med aktiv tuberkulossjukdom.

Vidare önskade vi utröna huruvida ökad resistens hos tuberkulosebakterien påverkar behandlingsutfallet. I en nationell studie med alla fall av MDR-tbc från 1992-2014 (nr= 158) fann vi att ökad grad av bakteriell resistens, mätt som den minsta hämmande koncentrationen, var associerat till en ökad risk för död, utebliven läkning samt återfall av tuberkulos (**Studie III**). Även stigande ålder samt diabetes var riskfaktorer för sämre behandlingsutfall av tuberkulos. Gällande behandlingen av MDR-tbc fann vi att läkemedlet pyrazinamid till rätt patienter, där resistensbestämning visat känslighet hos bakterien för pyrazinamid, var förknippad med förkortad längd av smittsamhet hos lungsjuka MDR-tbc patienter. Studien tillförde viktiga kliniska kunskaper att beakta vid behandlingen av MDR-tbc.

Graden av bakteriell resistens studerades också i **Studie IV**, där även läkemedelskoncentrationer i blodet uppmättes hos tuberkulospatienter med känslig tuberkulos från två svenska sjukhus (nr=31). Låga läkemedelskoncentrationer av förstahandsläkemedlen mot tuberkulos var vanliga (16-42 %), trots rekommenderade doseringar av medicinerna. I synnerhet noterade vi låga nivåer i blodet av det vitala läkemedlet rifampicin, (13/31 patienter, 42 %), vilket i tidigare studier har medfört ökad risk för sämre behandlingsutfall och resistensutveckling. Behandlande läkare bör vara uppmärksam på att läkemedelskoncentrationer kan behöva kontrolleras för att säkerställa adekvat läkemedelsexponering. Trots de låga läkemedelskoncentrationerna var behandlingsresultaten goda (100 % utläkning initialt, dock två återfall), möjligtvis pga. den låga graden av resistens hos tuberkulosebakterierna. Då behandlingen av resistent tuberkulos är ännu mer komplicerad då en kombination av minst fem läkemedel krävs har vi genomfört en liknande studie i Kina var vi undersöker läkemedelskoncentrationerna vid behandlingen av MDR-tbc där resultaten väntas nästa år. Sammanfattningsvis tyder vår forskning på att behandlingen av tuberkulos bör individualiseras, genom att graden av resistens, läkemedelsexponeringen i blodet samt kliniska faktorer som exempelvis diabetes och annan samsjuklighet tas i beaktning.

Under de fyra åren av mina doktorandstudier har mer än 40 miljoner människor nyinsjuknat i tuberkulos. I denna avhandling beskrivs olika strategier för att försöka förbättra behandlingen och överlevnaden av tuberkulos, men om vi ska nå Världshälsoorganisationens mål om att utrota sjukdomen till 2050 behövs större, globala satsningar inom folkhälsa och sjukvård. För att svara Skorpan i Bröderna Lejonhjärta, ingen ska behöva dö av denna behandlingsbara sjukdom, varken gammal eller ung!

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- II. **Davies Forsman L**, Giske CG, Bruchfeld J, Schon T, Jureen P, Angeby K. **Meropenem-clavulanic acid has high *in vitro* activity against multidrug-resistant *Mycobacterium tuberculosis*.**
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- III. **Davies Forsman L**, Jonsson J, Wagrell C, Werngren J, Mansjö M, Wijkander M, Groenheit R, Hammar U, Giske CG, Schön S, Bruchfeld J. **Minimum inhibitory concentrations versus clinical outcome in MDR-TB patients - a nationwide Swedish cohort study over two decades.**
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CONTENTS

1	Preface	1
2	Introduction	3
2.1	Tuberculosis – an overview	3
2.1.1	Tuberculosis epidemiology worldwide	3
2.1.2	Clinical aspects of TB	4
2.1.3	The pathogen – <i>M.tuberculosis</i>	4
2.1.4	Treatment of susceptible TB.....	5
2.1.5	Principles of <i>M.tuberculosis</i> resistance	6
2.1.6	The MDR-TB epidemic – TB resurges	6
2.1.7	Diagnosis of TB and MDR-TB – “going for the bug”	8
2.2	Drug resistance in <i>M.tuberculosis</i>	8
2.2.1	Defining resistance in TB	8
2.2.2	Methods of drug susceptibility testing.....	9
2.2.3	Minimum inhibitory concentrations – quantification of resistance	11
2.3	Treatment of TB - recommended doses and duration under revision.....	12
2.4	Treatment of MDR-TB – optimization urgently needed.....	12
2.4.1	Shortened treatment of MDR-TB with more drugs – the MDR-TB short course.....	14
2.4.2	Repurposed drugs – an untapped potential?.....	14
2.4.3	Surrogate markers of treatment outcome and clinical improvement of TB used in trials and clinical practice.	15
2.5	Pharmacokinetics and pharmacodynamics	16
2.5.1	Pharmacokinetics	16
2.5.2	How is pharmacokinetics of drugs studied?.....	16
2.5.3	Pharmacodynamics	17
2.5.4	PK/PD relationships	17
2.5.5	Pharmacometric modeling	17
2.6	Therapeutic drug monitoring (TDM) – integrating interactions of host, pathogen and drug	18
2.6.1	Therapeutic drug monitoring in TB.....	19
2.6.2	New treatment strategies for MDR-TB needed	19
3	Aims.....	25
3.1	Overall aim of the thesis.....	25
3.2	Specific aims of the included studies.....	25
4	Material and methods	26
4.1	Material / Study design and settings	26
4.1.1	Studies I & II	26
4.1.2	Study III.....	26
4.1.3	Study IV.....	26
4.2	Methods of drug susceptibility testing (DST)	27

4.2.1	Solid LJ media – Study III	27
4.2.2	BACTEC 460 TB and BACTEC MGIT 960 – Study III & IV	28
4.2.3	Drug susceptibility testing of pyrazinamide - Study III & IV	28
4.3	Minimal inhibitory concentrations – solid, liquid or microtitre methods	28
4.3.1	Solid media Middlebrook 7H10 - Study I & II	28
4.3.2	Intracellular versus extracellular MIC testing – Study I	29
4.3.3	MIC testing using BACTEC MGIT 960 - Study I, III & IV	29
4.3.4	Microtitre Sensititre™ plates – Study III & IV	29
4.4	Whole genome sequencing (WGS)	30
4.5	Drug concentration measurements	30
4.6	Epidemiology	30
4.6.1	Cohort study design	30
4.6.2	Random error	30
4.6.3	Bias	31
4.7	Statistical considerations	31
4.8	Ethical considerations	32
5	Results	33
5.1	Study I	33
5.2	Study II	35
5.3	Study III	36
5.4	Study IV	39
6	Discussion	41
6.1	Major findings	41
6.2	General discussion	41
6.2.1	The quest for new drug regimens – in with the new, in with the old	41
6.2.2	Reliable drug susceptibility and MIC-testing do matter	42
6.2.3	Therapeutic drug monitoring – a torch in the dark	45
6.2.4	One size does not fit all – the need to individualise therapy	49
7	Clinical implications and conclusions	51
8	Future perspectives	52
9	Acknowledgements	54
10	References	57

LIST OF ABBREVIATIONS

AUC	Area under the concentration versus time curve
BSL-3	Bio-safety level 3
CFU	Colony-forming units
C _{max}	Peak drug concentration
C _{2h}	Plasma drug concentration 2 h after drug intake
CFR	Cumulative fraction response
CRyPTIC	Comprehensive Resistance Prediction for Tuberculosis: an International Consortium
DM	Diabetes mellitus
DST	Drug susceptibility testing
EBA	Early bactericidal activity
ECOFF	Epidemiological cut-off
EMB	Ethambutol
HIV	Human immunodeficiency virus
INH	Isoniazid
LJ	Löwenstein-Jensen
LPA	Line-probe assay
MEM-CLA	Meropenem-clavulanic acid
MGIT	Mycobacterium Growth Indicator Tube
MDR-TB	Multidrug-resistant tuberculosis
<i>M.tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
NA	Not applicable
PCR	Polymerase chain reaction
PK/PD	Pharmacokinetic/Pharmacodynamic
PTA	Probability of target attainment
RCT	Randomised controlled trial
RIF	Rifampicin
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TDM	Therapeutic Drug Monitoring
TMP-SMX	Trimethoprim-sulfamethoxazole

TTP	Time to positivity (detectable growth of <i>M.tuberculosis</i> in liquid culture)
WHO	World Health Organization
WGS	Whole genome sequencing
XDR-TB	Extensively drug-resistant tuberculosis

1 PREFACE

“Please don’t transfer me to the TB-ward – it’s a death sentence!”

The 26-year old father of two pleaded with me not to transfer him from the medical ward to the tuberculosis (TB) ward, now that his TB diagnosis had been confirmed. His fears were not unjustified. Due to lack of isolation rooms and months of delays in receiving results of susceptibility patterns, highly resistant *M.tuberculosis* strains spread in the ward and mortality was staggeringly high.

This was the reality in 2007 in COSH hospital, Tugela Ferry, South Africa. During my year as a Senior Medical Officer in the hospital, I witnessed the extremely high mortality rate (96%) that has been reported from the hospital amongst multidrug-resistant TB patients co-infected with HIV (1). Most of the patients were of a similar age to me when they died. This experience has profoundly motivated my choice of career and research field.

There were many factors contributing to the high TB mortality in the region, such as delays in diagnosis, high levels of HIV comorbidity, poor logistics, overwhelmed health services as well as weak drug regimens. Although new highly effective drugs have recently become available against MRD-TB, there are already reports of acquired drug resistance. Eleven years later I can say that the solution is not just new, effective drugs; we need to take better care of the drugs we have. We need more knowledge about the intricate relationship between host, pathogen and drug.

A few weeks later I went to visit the same patient in the TB-ward, only to find that he had passed away. A week later, the DST result finally became available and revealed that none of the drugs he had been treated with were effective.

2 INTRODUCTION

2.1 TUBERCULOSIS – AN OVERVIEW

“Where youth grows pale, and spectre-thin and dies”

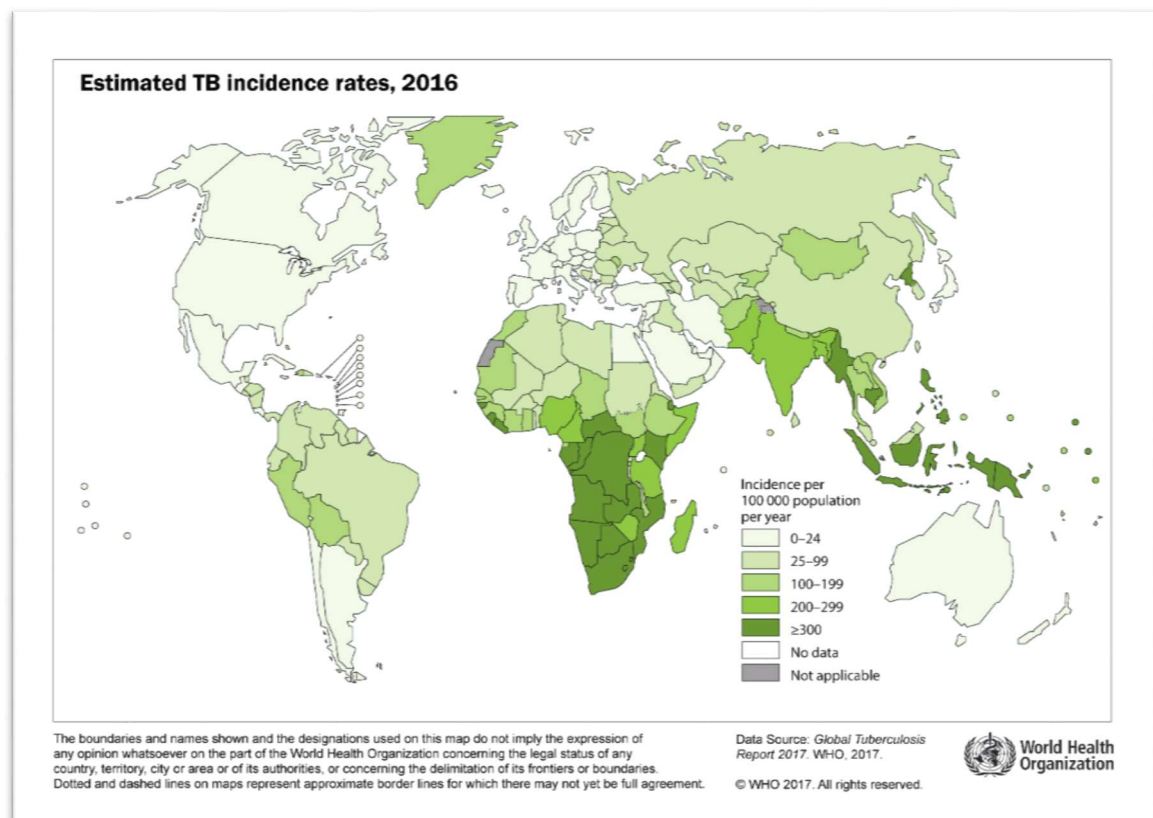
“Ode to a Nightingale”, by John Keats, who died at the age of 26 of TB.

The historic, almost romantic, notion of tuberculosis (TB) has since long been surpassed by stigma. TB, caused by the bacteria *Mycobacterium tuberculosis* (*M. tuberculosis*), was for a long time seen as a disease of the deprived, due to its rapid spread in poor, malnourished people. In recent decades, the stigmatisation has been exacerbated by the association with HIV (2). TB had a substantial impact on Swedish society during the 18th to early 20th century, with an incidence in 1940 of 300/100 000 inhabitants, comparable to the current incidence of TB in South Africa. Since the late 1940s, there has been a dramatic drop in TB incidence in Sweden to approximately 5 cases/100 000 a year (3). Nevertheless, TB is still a global health emergency world-wide.

2.1.1 Tuberculosis epidemiology worldwide

TB is now the leading infectious disease killer as an infective agent, surpassing HIV/AIDS and malaria combined. There were an estimated 10 million new cases in 2017 and distribution world-wide is shown in Figure 1 (4).

Figure 1. Global incidence of tuberculosis 2016.



The incidence of TB is decreasing roughly 2% annually, whereas the TB mortality rate is falling approximately 3% per year globally. Despite this decrease, there were more than 3500 casualties per day worldwide in 2016 due to TB. The annual death toll of TB is close to 1.6 million people (4). Over 95% of all TB deaths occur in resource-limited countries, although the total death rate of TB worldwide has almost halved from 1990 to 2015 (5). Currently, around 16% of patients with TB die from their disease. The World Health Organization (WHO) End TB Strategy aims for a 95% reduction in TB deaths and a 90% reduction in new TB cases from 2015 to 2035 with the goal of TB elimination. The decrease in new TB cases and mortality has to be substantially accelerated if these goals are to be achieved. The global treatment success rate of TB is 83%, close to the WHO goal of >85% (6). However, a major obstacle against TB control and elimination is the estimated one-third of all active TB cases world-wide that never get diagnosed or reported (4).

2.1.2 Clinical aspects of TB

As an airborne infection, TB is a social disease, affecting mainly household and close contacts. An infectious TB patient typically infects around 3-6 people per year and is not as highly contagious as other air-borne pathogens, such as the measles virus (7). After infection, individuals may develop so-called latent TB infection, defined as a positive tuberculin skin test or interferon-gamma release assay (IGRA) without symptoms and signs of TB, including a normal chest x-ray (8). Latent TB can reactivate years later and the risk increases with immunosuppression or the use of immunosuppressant drugs such as TNF- α inhibitors. Indeed, HIV infection, in particular untreated, is the strongest risk factor for TB disease (9). However, malnutrition is more common in absolute numbers and thought to be attributed to 27% of TB cases worldwide (10). The individual's immune status is paramount in controlling *M.tuberculosis*, with only 5-10% of infected people developing active TB during their lifetime. Upon inhalation, *M.tuberculosis* is phagocytosed by resident lung macrophages in which they may replicate due to active blockage of the fusion with the lysosome, ensuring its survival (11). After an incubation time of 6-8 weeks, the classic symptoms of pulmonary TB (PTB) are prolonged cough, night-sweats, weight-loss and fever, although approximately 25% of TB patients have no or few symptoms (12). In the natural course of TB before effective treatment with antibiotics, up to 25% of people with active TB will be cured of the disease, whereas around 50% will die and the rest develop chronic disease (13). Old granulomas can be seen as calcified scars on a chest x-ray, where *M.tuberculosis* can lie dormant, metaphorically in calcified sarcophaguses, while awaiting an opportunity to resurge (14).

2.1.3 The pathogen – *M.tuberculosis*

M.tuberculosis is a slow-growing aerobe rod-shaped facultative intracellular bacteria, characteristically acid-fast when microbiological staining techniques are used (15). The *M.tuberculosis* complex consists of the genetically linked *M.tuberculosis* and rare causes of TB disease such as *M.africanum*, the zoonotic *M.bovis*, *M.microti* and *M.caprae*. The thick, lipid-rich cell wall makes the bacteria resilient to drying. *M.tuberculosis* can thrive extra- and intracellularly, extracellularly early on or in disseminated disease but typically resides inside the macrophage (16). Activated macrophages, T lymphocytes as well as tumour necrosis factor (TNF- α), interleukin-12 and interferon- λ are vital for the formation of a granuloma,

with the aim of killing and preventing dissemination of the bacteria (17). In the granuloma's harsh conditions with anoxia and depleted nutrients, the replication is slowed. A further step is the complete halt of replication without bacterial death, when the bacteria enters a dormant stage and can remain viable without causing active disease. Later immunosuppression can lead to reactivation years later, where dormant bacilli multiply, causing active TB (18).

Figure 2. Hypothetical model of three subpopulations of *M.tuberculosis*, depending on growth.

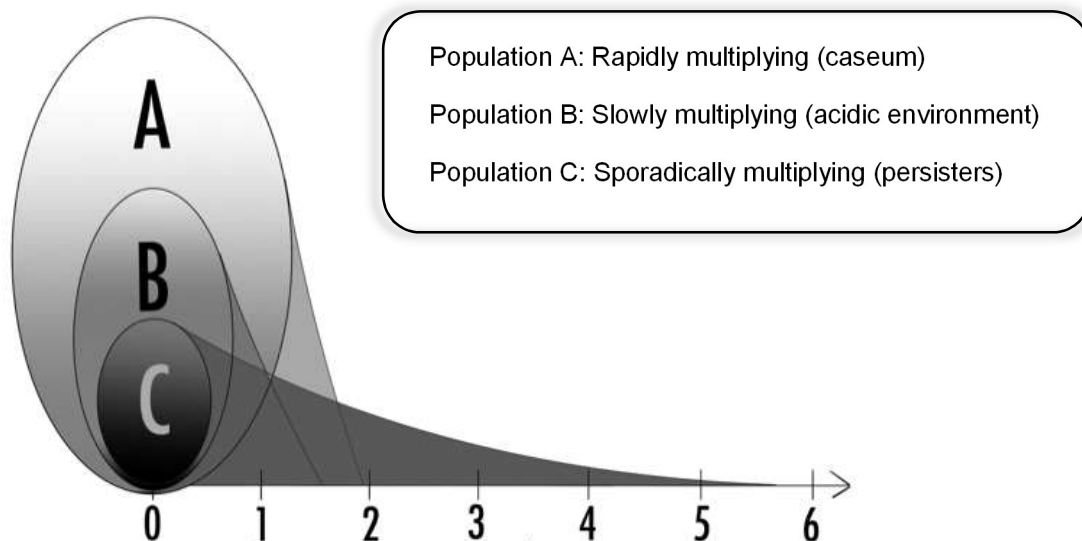


Illustration by Paul Garbers adapted from 'A clinician's guide to tuberculosis' Iseman 2000.

The immune system can often control bacterial growth by an effective cell-mediated immune response, but cannot always eradicate bacilli in the slowly replicating phase or even dormant bacteria, known as persisters, a major cause of relapse and reactivation (Figure 2) (19). Sterilizing activity of a drug refers to the killing of the last viable bacteria, such as persisters and slowly multiplying bacilli (20, 21).

2.1.4 Treatment of susceptible TB

The scientific efforts in the 40s and 50s to find a cure for TB finally resulted in the ground-breaking turning point where TB changed from a deadly to a curable disease (22).

Unfortunately, as a result of monotherapy, resistance soon emerged and it became evident that a combination of drugs was mandatory (23). Standard treatment of TB involves an intensive phase of two months with the first line drugs, rifampicin, isoniazid, pyrazinamide and ethambutol, followed by a continuation phase of four months with only rifampicin and isoniazid.

The drug regimen is designed to kill all different subpopulations of *M.tuberculosis* isolates (Figure 2). *M.tuberculosis* is killed bi-phasically, where fast-replicating bacteria are rapidly killed by the highly bactericidal isoniazid, whereas persisters and slowly replicating bacteria require drugs with sterilising activity such as rifampicin and pyrazinamide, as well as long

treatment periods to avoid relapse (24-26). The lipophilic nature of rifampicin allows activity of intracellular bacteria (27). Pyrazinamide also has good penetration and activity in the acidic environment in caseating granulomas (20, 28).

Together, the first-line drugs target different subpopulations of *M.tuberculosis* with varying metabolic activity and location (29) with isoniazid being most effective against rapidly dividing bacteria, pyrazinamide most effective in acidic environments and rifampicin most effective as a sterilising agent of persisting bacteria (Figure 2).

2.1.5 Principles of *M.tuberculosis* resistance

The main mechanism of drug resistance in *M.tuberculosis* is spontaneous chromosomal mutations. These mutations will result in resistance through drug target alterations, overexpression of drug target, disruption of prodrug activation (for example katG and isoniazid) and the activation of efflux pump (30). In a bacterial population, mutations occur naturally at a low rate (10^8 for rifampicin and 10^6 for isoniazid) and are unlikely to multiply in a well-designed multi-drug TB therapy (31). Thus, acquired drug-resistance in *M.tuberculosis* is primarily a man-made phenomenon that develops mainly through low drug-exposure in the patient due to, for example, underdosing, poor adherence, poor quality drugs, irregular drug supply or poor drug absorption (29, 32).

2.1.6 The MDR-TB epidemic – TB resurges

The WHO End TB Strategy aims for a world free of TB in 2050 (33). Although susceptible TB is slowly decreasing worldwide, the reduction rate is too slow (2%) and MDR-TB are increasing (6). MDR-TB and extensively drug-resistant tuberculosis (XDR-TB) are defined in Table 1.

Table 1. Definitions of drug-resistant tuberculosis.

RR-TB*	<i>M.tuberculosis</i> resistant to rifampicin
MDR-TB	<i>M.tuberculosis</i> resistant to rifampicin and isoniazid
pre-XDR-TB	MDR-TB with additional resistance to any fluoroquinolone (ofloxacin, moxifloxacin, levofloxacin) OR injectable drug (capreomycin, kanamycin, amikacin)
XDR-TB**	MDR-TB plus resistance to: any fluoroquinolone (ofloxacin, moxifloxacin, levofloxacin) AND any injectable drug (capreomycin, kanamycin, amikacin)

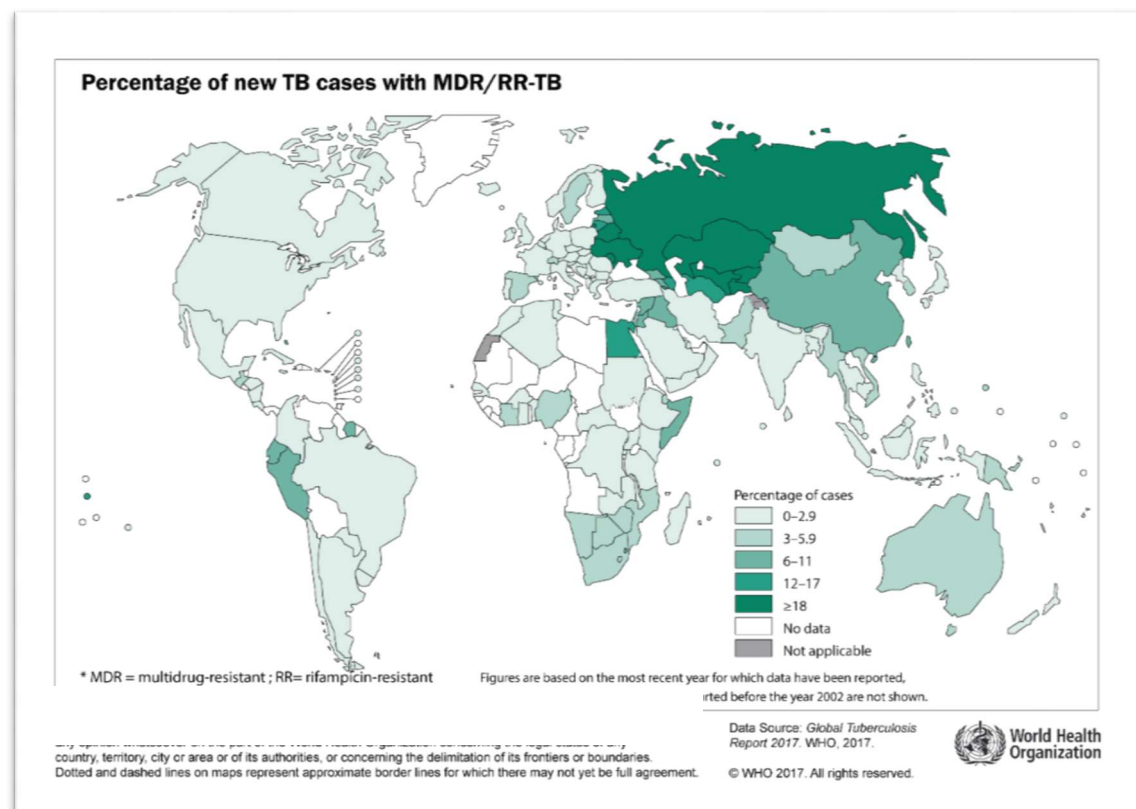
* The same recommended treatment as for MDR-TB according to WHO

* Definition of XDR-TB likely to change due to updated treatment recommendations where second-line injectable drugs have been downgraded.

WHO estimates that 558 000 new MDR/RR-TB cases emerged in 2017 alone, of which the vast majority (71%) were not diagnosed or reported and only a quarter was started on treatment (4). As drug resistance is increasing, drug susceptibility testing (DST) is needed for accurate treatment of TB, but is often not performed in many high-burden countries. In many settings, only sputum from retreatment TB-patients receive primary DST. However, the fact that 38% of Belarus patients without previous TB treatment were found to have MDR-TB in 2016 (6), exemplifies the need of initial DST for all patients, in order to avoid missing drug-resistant TB. Even if MDR-TB is identified, only 36% of MDR-TB patients receive DST for second-line drugs (34).

Almost half of the world's burden of MDR-TB is found in India, China and the Russian Federation (4) (Figure 3). Although Europe has less than 5% of all TB, it has a disproportionate 25% of the total burden of MDR-TB in the world. The number of MDR-TB patients in Sweden, although still low around 3%, is increasing as a reflection of the MDR-TB epidemic at large (35).

Figure 3. World-wide distribution of MDR-TB 2017.



2.1.7 Diagnosis of TB and MDR-TB – “going for the bug”

Rapid and correct diagnosis of TB is vital to achieve infection control. *M.tuberculosis* is detected by smear microscopy and by PCR, including point-of-care tests. Mycobacterial culture requires technical expertise as well as a well-equipped biosafety level 3 (BSL-3) laboratory and is not widely available. Smear microscopy positivity is a marker of high infectiousness, with more than 5,000-10,000 bacteria/ml of sputum required for visual detection (36). The use of PCR for the detection of *M.tuberculosis* greatly increases the sensitivity for sputum samples, from 50% with smear microscopy to around 70-95% with PCR, depending on bacterial load (37, 38) as compared to culture. Culture to detect *M.tuberculosis* remains as a reference method and the solid egg-based Löwenstein-Jensen (LJ) is the most commonly used media worldwide. The introduction of liquid broth systems such as BACTEC in the 1980s, reduced the mean time-to-detection of positive growth to 1-2 weeks for smear positive samples, compared to 3 weeks on solid media (39).

2.2 DRUG RESISTANCE IN *M.TUBERCULOSIS*

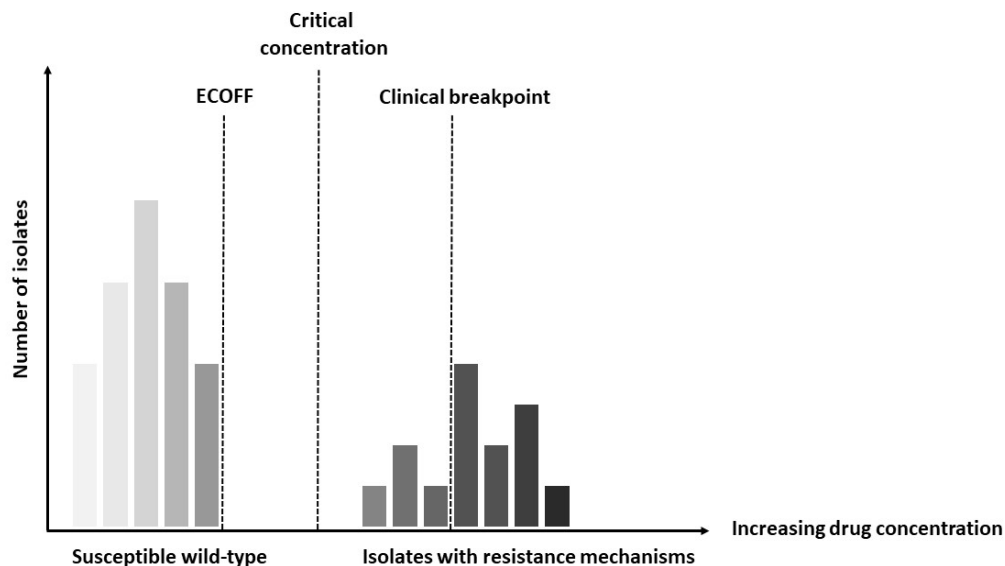
2.2.1 Defining resistance in TB

In every *M.tuberculosis* population there is heterogeneity and a number of strains will, by spontaneous mutations, differ from the rest of the bacteria by their ability to grow, despite the presence of an active antibiotic. Depending on the extent of the proportion of resistant subpopulations, this might affect clinical outcome. Therefore, resistance is defined as more than 1% of growth in the presence of the critical concentration of a drug (40).

The critical concentration has recently been redefined by WHO as “the lowest concentration of an anti-TB agent *in vitro* that will inhibit the growth of 99% (90% for pyrazinamide) of phenotypically wild-type strains of *M.tuberculosis* complex” (40). Critical concentrations are method-specific and should ideally separate the wild-type population from the non-wild-type. Overlapping distributions of the two populations in combination with high methodological variability of some drugs decreases DST reproducibility, as seen for ethambutol (41). The epidemiological cut-off (ECOFF) is the highest MIC within the wild-type population, i.e. encompassing >99% of isolates lacking phenotypic resistance (Figure 4) (40).

A clinical breakpoint should separate strains that are likely to respond, as opposed to being unlikely to respond to treatment, and should be defined by considering the ECOFF, in relation to antimicrobial exposure and microbial kill, as well as clinical outcome (42). Unfortunately, there are few defined clinical breakpoints for TB-drugs and critical concentrations are often used, since extensive clinical outcome data are lacking (40, 43, 44).

Figure 4. Schematic illustration of the relationship between ECOFF, critical concentration and clinical breakpoint. The critical concentration and the clinical breakpoint (if defined) regularly coincide. The number of MIC dilution steps has been increased for clarity.



As Canetti eloquently stated already in 1963, resistance is defined in a laboratory environment and does not automatically correspond to clinical treatment failure. Rather, the presence of *M.tuberculosis* resistance *in vitro* should be viewed as a decreased probability of treatment success *in vivo* (45), in agreement with other bacteria.

2.2.2 Methods of drug susceptibility testing

Mycobacterial resistance can be measured by molecular or phenotypic methods. DST is mainly performed using the indirect method, such as from cultured isolates. Direct DST, i.e. from sputum samples (smear and/or PCR positive), is sometimes used for molecular methods such as line-probe assays (LPAs).

2.2.2.1 Molecular DST – to rapidly rule in, but not rule out resistance

Molecular methods use DNA sequences to detect known mutations linked to phenotypic resistance. The mutations might consist of single nucleotide polymorphisms (SNPs), deletions or insertions and genomic rearrangements, giving rise to low-level or high-level phenotypic resistance (46). There are different molecular methods in clinical practice to identify resistance in *M.tuberculosis*; the Xpert MTB/RIF and LPA. The semi-automated Xpert MTB/RIF detects mutations in the genetic region *rpoB* (encoding RNA polymerase) linked with phenotypic resistance against rifampicin with high sensitivity and specificity (94% and 98% respectively) in under 2 hours directly from sputum samples, without requiring a biosafety laboratory (47). The improved Xpert MTB/RIF Ultra has a marked increased sensitivity; a 17% increased sensitivity for smear-negative patients and 13% increase for paucibacillary HIV patients and similar specificity compared to Xpert MTB/RIF. A related investigational assay with DST for major second-line drugs is being explored (48).

Rifampicin is commonly used as a marker of MDR-TB since simultaneous resistance to isoniazid is seen in more than 90% of cases (49). Moreover, WHO recommends MDR-TB treatment also for disease due to rifampicin mono-resistant isolates (50). GeneXpert is suitable in settings with a high prevalence of rifampicin resistance as well as for diagnostic purposes in low-endemic settings (51).

LPAs are more labour-intensive, requiring cultured *M.tuberculosis* as well as a TB-laboratory but can also detect resistance mutations for isoniazid, second-line injectable drugs and fluoroquinolones within 8 hours (52). LPAs show varying performance depending on drug; 99% sensitivity for isoniazid and 85.6% for fluoroquinolones from indirect samples (53). Molecular methods can greatly speed up the detection of resistance, but they still show a relatively low negative predictive value for many drugs, such as pyrazinamide, as many resistance mutations are unknown (54, 55). Consequently, they should presently only be used to rule in, rather than rule out resistance.

2.2.2.2 Whole genome sequencing – for mutation detection and strain identification

Whole genome sequencing (WGS) is a method to establish the full nucleotide sequence of an organism's DNA (i.e. genome) and can be used for TB diagnosis, mutation detection and strain identification for epidemiological purposes. The technique has been shown to shorten the average time to result from 3 weeks with conventional DST, to 9 days using WGS at a lower cost (56). However, WGS is only available in specialised centres and the relationship between mutations and phenotypic resistance is not completely known. For instance, about 10% of phenotypically isoniazid resistant strains show no mutation in either *katG* or *inhA*, indicating mutations in other regions or alternative resistance mechanisms. Conversely, a mutation does not lead to resistance unless it is expressed. (30). Furthermore, it remains technically difficult to perform WGS directly from sputum samples, adding time for *M.tuberculosis* culture to the turn-around time of the method. Nevertheless, WGS has currently replaced phenotypic DST for isolates without resistance mutations in England and the Netherlands (57). There are ongoing efforts supported by the Bill & Melinda Gates foundation to enable the interpretation of WGS data for TB in a comprehensive open-access database (58, 59), although smaller databases already exist (ReseqTB, TBResist etc). In a recent large study by the CRYPTIC consortium, susceptibility to first-line drugs could be correctly predicted by DNA sequencing (97.9% match) (60).

2.2.2.3 Phenotypic drug susceptibility testing

DST is traditionally performed on either an egg- or agar-based medium using one of three different methods; the critical concentration, resistance ratio and the proportion method. DST can be performed with direct (sputum) or indirect samples (cultured *M.tuberculosis* isolates) on solid (eg. LJ) or liquid media (eg. 7H9). The use of liquid media (7H9) has shortened the turnaround time for DST for pre-cultured isolates, from 3-4 weeks on solid media to approximately 1-2 weeks using liquid media with BACTEC MGIT 960 (61).

The most commonly used method of susceptibility testing for *M.tuberculosis* is the 1% indirect agar proportion method, where the percentage of growth of a defined inoculum in a culture media containing the critical concentration of the drug is compared to growth in a drug-free control (45, 62, 63). Critical concentrations have only been defined for the indirect

proportion method. DST is typically performed in BACTEC MGIT 960 in resource-rich settings. DST of pyrazinamide is often not performed in many high-burden countries, due to the requirement of specialised, slightly acidic media during culture and problems with reproducibility and reliability (64).

Table 2. Overview of phenotypic drug susceptibility testing in *M.tuberculosis*. Adapted from previously published review (65).

Method	Detection of growth	
	Direct evidence	Indirect evidence
Critical concentrations	Resistance ratio	Nitrate reductase assay
	Absolute concentration	Resazurin test
	MODS	Alamar blue test
	Microtitre plates (e.g. Sensititre)	
Critical proportion and critical concentrations	Löwenstein-Jenssen solid media	BACTEC MGIT 960 (7H9)
	7H10 or 7H11 (CLSI protocol)	

MODS: Microscopy observed direct susceptibility testing, CLSI: Clinical and Laboratory Standards Institute, MGIT: Mycobacterial growth indicator tubes

2.2.3 Minimum inhibitory concentrations – quantification of resistance

The minimum inhibitory concentration (MIC) for tuberculosis is the lowest concentration that inhibits >99% or visible growth of a microorganism (liquid/solid media) (40). A range of concentrations in two-fold serially diluted steps are tested and the MIC values typically form a normally distributed curve. Although more time-consuming, the MIC provides a more descriptive level of resistance to the drug than other phenotypic DST methods.

MIC determination can be performed on solid media, such as LJ or Middlebrook 7H10 and there is no reference method for MIC determination. More commonly liquid media, such as BACTEC MGIT 960, is used to reduce turn-around time. It is clinically important to be aware that MIC determination is associated with inter- and intra-laboratory variability (66).

Recently, prefilled broth-based microtitre plates with freeze-dried antibiotics have enabled simultaneous MIC determination of 12-14 first- and second-line drugs. Mycobacterial suspensions are inoculated in the 96-well plate, incubated and read after 10-21 days (67). The advantages of the microtitre plate are reduced cost when testing multiple drugs compared to BCTEC MGIT, as well as it being less labour-intensive. The disadvantages are manual reading with truncated MICs and the need of a BSL 3 laboratory, decreasing its feasibility in resource-limited settings. Furthermore, MIC testing of pyrazinamide is not yet possible in microtitre plates. To date, there are no defined critical concentrations for microtitre plates (67). Critical concentrations for the agar proportion method on solid culture media have been suggested for the Sensititre plate but need validation (68).

Knowledge of bacterial resistance (DST/MIC) is instrumental to guide management of individual patients.

2.3 TREATMENT OF TB - RECOMMENDED DOSES AND DURATION UNDER REVISION

The current recommended drug regimen of TB is under scrutiny, especially the dose of rifampicin, but also levofloxacin and pyrazinamide (NCT01918397) (69). In the 1960s, the dose was selected due to fear of toxicity but also due to costs; for example, one week of monotherapy with rifampicin was equivalent in cost to the whole six-month treatment of today (70). There is evidence of subtherapeutic blood levels of rifampicin mainly from high-endemic countries, whereas fewer studies have been performed in low-endemic settings with high-quality drugs.

The treatment length of TB should ideally be shortened to reduce costs, side effects, drug burden and risk of resistance developing. Unfortunately, four clinical trials have failed to show support for a shortened four month therapy through the addition of a fluoroquinolones (71-74). The cure rate of susceptible TB in Sweden is similar to the global success rate of 82% reported by WHO (4, 35). Relapse of TB after successful treatment is seen in less than 1-2% of patients in high-resource settings (35, 75). Failure of TB treatment is multifactorial, but it is important to exclude acquired resistance. A vital paradigm in TB treatment is never to add a single drug to a failing therapy to avoid further resistance to develop. A repeat DST should always be performed if clinical improvement is slow.

2.4 TREATMENT OF MDR-TB – OPTIMIZATION URGENTLY NEEDED

MDR-TB is difficult to treat, requiring longer treatment durations due to less efficacious drugs, and is associated with increased cost, adverse events and poor treatment outcome. To enable the programmatic treatment of MDR-TB, especially at a lower health system level, a standardised MDR-TB treatment is common, although WHO also supports an individualised treatment, guided by DST and contact history (50).

Unfortunately, treatment outcome of MDR-TB and especially XDR-TB is approaching the pre-antibiotic era, as the success rate of XDR-TB in Europe 2016 was 33.8% (cohort from 2013) (76) and a global cure rate of 55% of MDR-TB (4). However, there is hope for improved cure rates with the long-awaited addition of two new highly effective drugs; bedaquiline and delamanid (Table 3).

Table 3. New approved antituberculosis drugs.

Generic name	Brand name	Chemical class	Mechanism of action on <i>M.tuberculosis</i>
Bedaquiline	Sirturo	Diarylquinoline	Inhibits energy metabolism by blocking ATP-synthase
Delamanid	Delytba	Nitroimidazole	Inhibits cell wall by blocking the synthesis of mycolic acids

The use of one of the two additional drugs, bedaquiline, has recently been upgraded in the newly revised WHO treatment recommendations of MDR-TB, forming an all oral background regimen treatment with a fluoroquinolone and linezolid (77). The basis of this change partly stems from a recent individual patient meta-analysis with 12 030 patients included in studies from 2009-2016, where more than 61% had a successful treatment outcome. The optimal number of drugs were found to be five in the initial phase and four in the continuation phase, with the mean optimal treatment duration being 21 months (78).

Table 4. Recommended drugs for MDR-TB treatment – 2018 WHO update

Group	Drug	Mechanism of action	Adverse drug reaction
A (include all if possible)	Moxifloxacin/ Levofloxacin	Inhibits DNA gyrase	Arthralgia, achilles tendon rupture
	Bedaquiline	Inhibits DNA synthase	QT-prolongation, liver toxicity
	Linezolid	Inhibits protein synthesis	Pancytopenia, polyneuropathy
B (add both if possible)	Clofazimine	Phenazine die, interfering with DNA synthesis	Reversible skin discolouration
	Cycloserine OR Therizidone	Prevents cell wall synthesis	Neurotoxicity, psychiatric disturbances, neuropathy
C add when drugs from Group A and B cannot be used)	Ethambutol, Delamanid, Pyrazinamide, Imipenem-cilastatin OR Meropenem/clavulanic acid, Amikacin, Ethionamide/Prothionamide, <i>p</i> -aminosalicylic acid		

Adverse drug reactions caused by second-line drugs are very common, as high as 79% in previous studies (79). A Cochrane review revealed that as many as 16-49% of patients with MDR-TB fail to complete treatment (80). Hopefully, this will change with the newly recommended and entirely oral treatment, although adverse drug reactions are frequently seen with long-term use of linezolid and monitoring is difficult in high-endemic settings (81).

2.4.1 Shortened treatment of MDR-TB with more drugs – the MDR-TB short course

Ideally, MDR-TB treatment should be a short and completely oral regimen. The so-called Bangladesh regimen for MDR-TB consists of a 9-month treatment course with an initial regimen of seven anti-TB drugs, kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, high-dose isoniazid and ethambutol for 4-6 months, followed by five months of moxifloxacin, clofazimine, pyrazinamide and ethambutol. This regimen attained a treatment success rate of more than 85% and with very few relapses in the two-year follow-up period (82). A slightly higher treatment success rate (89%) was seen in a 12-month MDR-TB regimen in Cameroon, although as many as 43% of the patients suffered from hearing impairment due to treatment with kanamycin (83). The MDR-TB short course regimen was endorsed in 2016 by WHO, although its use has been questioned in some settings due to widespread resistance to included drugs, such as pyrazinamide and ethambutol (84).

Interim data from the STREAM I trial evaluating the short-course regimen showed successful treatment outcome in 78.1% versus 80.6% using standard WHO MDR-TB treatment of 20-24 months (85), although non-inferiority has not yet been shown (86). The STREAM II trial is evaluating three short course regimens compared to standard MDR-TB treatment. Two of the regimens contain bedaquiline, of which one is all oral, which would greatly simplify treatment (NCT02409290).

2.4.2 Repurposed drugs – an untapped potential?

Evidence of resistance was seen not long after the introduction of bedaquiline and delamanid (87) and there are contraindications such as QTc-prolongation with risk of ventricular arrhythmias (both drugs) or hypoalbuminemia (delamanid) restricting their use. Alternative drugs are always needed, which is why the use of already approved drugs is being investigated (69). Repurposed drugs are drugs with activity against diseases other than they were originally intended for. An examples of a repurposed drug is linezolid, used for treatment of gram-positive infections. *In vitro* studies have shown anti-TB activity of trimethoprim-sulfamethoxazole (TMP-SMX), meropenem-clavulanic acid (MEM-CLA) as well as imipenem-cilastatin (88-90). However, the majority of these studies are performed on susceptible isolates and rarely include clinical MDR-TB strains. Moreover, there are no clinical trials evaluating the effect *in vivo* of these drugs.

2.4.3 Surrogate markers of treatment outcome and clinical improvement of TB used in trials and clinical practice.

In order to evaluate new drugs or drug regimens for TB, surrogate markers such as sputum culture conversion and changes in bacterial sputum load are often used. However, short duration trials are intrinsically limited in predicting the final treatment outcome (91) and valid biomarkers predicting treatment outcome are of importance.

2.4.3.1 Sputum culture conversion

Time to sputum culture conversion or sputum culture conversion after two months of treatment is commonly used to evaluate treatment effect in drug trials (92). However, the supporting evidence is weak and studies have shown sputum culture conversion to be an imperfect predictor of the final treatment outcome (93, 94), which is problematic for TB drug development. A systematic meta-analysis, treatment evaluation with sputum-smear microscopy and/or mycobacterial culture after two months of treatment showed low sensitivity and modest specificity for predicting failure and relapse of susceptible TB. The negative predictive value (93%) was substantially higher than the positive predictive value of sputum culture conversion (9-18%), meaning that a negative sputum culture at month two makes relapse and failure unlikely. However, a positive sputum culture at month 2 could not predict relapse and failure (95). Similar results have been shown for MDR-TB, where culture conversion at month 2 of treatment had a sensitivity of 27.3% (95% CI 16.6-41.4) in predicting successful treatment outcome (vs failure/death) in a study with 1712 patients. Sputum culture conversion at month 6 had a higher sensitivity (91.8%, 95% CI 85.9%-95.4%) but lower specificity (57.8%, 95% CI 42.5%-71.6%) (96).

2.4.3.2 Time to positive culture (TTP) and early bactericidal activity (EBA)

Early bactericidal activity (EBA) assesses the fall in colony forming units (CFU) on agar plates and is a measure of the bacterial burden. Daily sputum samples from smear-positive TB patients taking a particular drug is traditionally used to evaluate drug efficacy in TB treatment trials (97). However, EBA only assesses the initial phase of bacterial kill, as exemplified by clofazimine which has no effect on the EBA the first 14 days (98). Liquid media has higher sensitivity for mycobacterial growth (99) and time to culture positivity (TTP) in BACTEC MGIT 960 has been shown to be a useful alternative for assessment of drug efficacy (100, 101). TTP data can be easily accessed from the BACTEC MGIT 960.

2.4.3.3 Clinical severity scores – TBscore & TBscore II

Clinical severity scores, to increase case-finding and to predict treatment outcome, has been developed for TB. The TBscore II is a simplified version of the original TB-score, where physical examination of a physician was required. The variables included in TBscore II are cough, dyspnoea, chest pain, anaemia, body mass index (BMI) <18 and <16, mid upper arm circumference (MUAC) <220 mm and <200 mm. TBscore II has been validated in two high-burden cohorts (Ethiopia and Guinea-Bissau). A decrease of less than 25% of TBscore II from baseline to two months of treatment was associated with subsequent treatment failure in Guinea-Bissau (p=0.007) (102), but not in Ethiopia. Today, there is no clinical validation of the TB-score or other clinical scoring systems in low-endemic settings.

2.5 PHARMACOKINETICS AND PHARMACODYNAMICS

Pharmacokinetics (PK) and pharmacodynamics (PD) describe the interactions of drug and body.

2.5.1 Pharmacokinetics

The PK of a drug describe what the body does with the drug, i.e. how the drug is absorbed, distributed and cleared from the body. The changes of drug concentrations in the body over time are commonly summarised with the measures absorption (F), volume of distribution (V) and elimination (CL) (Table 5) (103).

Table 5. Definitions of key pharmacokinetic measures.

Absorption	A	Bioavailability, the unchanged fraction from 0-1 (0-100%) of the administered dose that reaches the circulation. Large, hydrophilic drugs are poorly absorbed as opposed to small, lipophilic drugs. First-pass metabolism by the liver might also lower the bioavailability.
Volume of distribution	Vd	The apparent volume that the drug is distributed in, relating the administered dose to the plasma concentration. The volume might be over 10 000 L (extensive tissue distribution). The volume of distribution is affected by protein binding where high protein binding gives a low V.
Clearance $CL = V / k$ $k = \text{constant}$ $CL = \text{rate of elimination} / C$	CL	The rate of drug elimination divided by the plasma concentration of the drug i.e. the volume of fluid cleared each unit of time (eg. L/h). The clearance determines the plasma concentration at steady state, i.e. the C_{average} . The renal clearance is decreased in case of acute renal failure.

2.5.2 How is pharmacokinetics of drugs studied?

In order to estimate the C_{max} and the AUC, multiple blood samples are needed. Only a few patients are needed in a so called rich-sampling scheme, with multiple blood tests from the time of the first dose and repeatedly thereafter. The AUC can then be calculated, using the trapezoidal method, where the area under the curve is divided into smaller trapeziums, so that the areas can be calculated and totalled. The fewer the samples, the less reliable is the estimation of the AUC. In order to study more patients, a limited sampling strategy, where two or three blood samples are enough to estimate the AUC, has been developed for many TB-drugs (104). Multiple samples are also needed to estimate the C_{max} , since delayed absorption might lead to a delayed peak in drug concentration.

It is the free, unbound concentration in plasma that is the active component of the drug (103) . The total drug concentration of the drug can be measured and the free (f) drug concentration can be estimated by multiplying with known constants of protein binding, if the free concentration is not measured directly in the plasma.

2.5.3 Pharmacodynamics

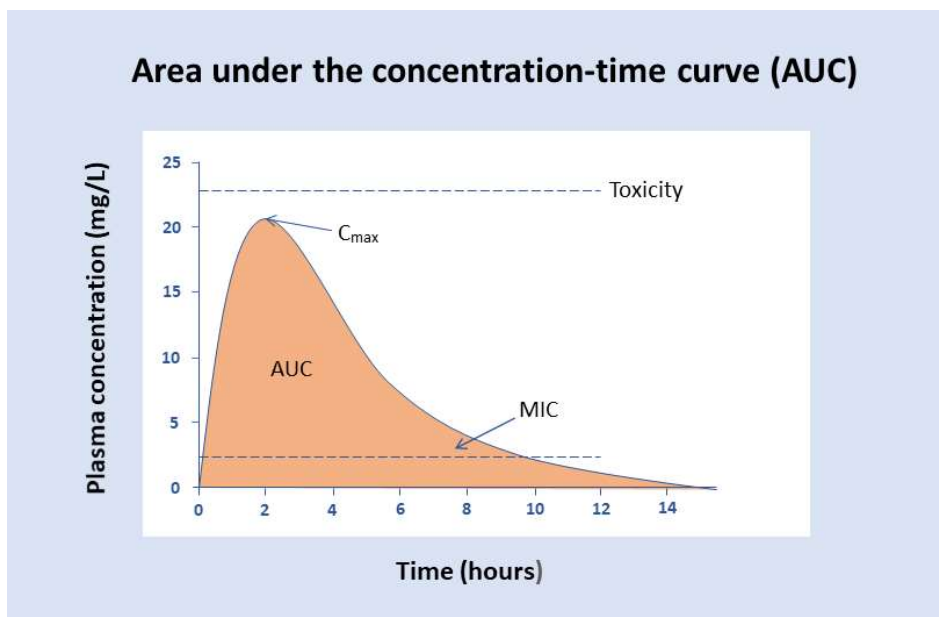
The PD of a drug describe what the drug does to the body, i.e. the effect the drug exposure has on the body or on the bacteria. This could be pain relief or microbial killing, for example.

2.5.4 PK/PD relationships

By investigating the relationship between the two parameters PK and PD, the optimal dose of a drug can be estimated, balancing effect and toxicity.

Firstly, the PK/PD relationship best describing the drug effect needs to be established and can be either concentration-dependent (C_{\max} , AUC/MIC) or time-dependent (%T>MIC). There is often a close relationship between the PK/PD indices C_{\max} /MIC and AUC/MIC. The optimal PK/PD index associated with best clinical outcome and suppression of drug resistance is then established.

Figure 5. Schematic illustration of the area under the concentration versus time curve (AUC), maximal concentration (C_{\max}) and minimum inhibitory concentration (MIC).



2.5.5 Pharmacometric modeling

"...all models are wrong, but some are useful." George E.P. Box 1987

Pharmacometry is a multi-disciplinary field where pharmacology, statistics, mathematics and physiology meet. By pharmacometric modelling, predictions of a drug's PK and PD characteristics can be made in a population, which is useful in assessing which dose will probably result in a desired outcome. Monte Carlo simulation is an example of a model useful for dose selection and breakpoint determination. Here, known PK parameters from the target

group are randomly selected, following the known standard deviation of a population and the responses of approximately 10 000 patients are then simulated, thus reducing the number of patients actually needed to be studied. The probability of target attainment (PTA) can also be calculated, i.e. the probability of achieving a certain PK/PD goal following Monte Carlo simulation for given MIC-values. The cumulative fraction response (CFR) calculates a PTA, while taking the local MIC distribution into account, and may vary between settings (105).

A clinical application of PK/PD and pharmacometry is therapeutic drug monitoring (TDM).

2.6 THERAPEUTIC DRUG MONITORING (TDM) – INTEGRATING INTERACTIONS OF HOST, PATHOGEN AND DRUG

The over-arching aim of TDM is to improve treatment outcome and reduce toxicity and adverse drug reactions. TDM involves measuring drug blood concentrations and adjusting dosages in order to optimise therapy and reduce adverse-effects. TDM integrates the concentration and the effect of a drug while taking the efficacy of drug and patient toxicity into account. Since the 1970s, TDM has been used to individualise therapy in many areas of medicine, such as immunosuppressant drugs. Monitoring of drug concentrations is especially important where there is a narrow therapeutic window (for example aminoglycosides) or when there is substantial inter-individual variation. The lowest drug concentration ensuring efficacy, and the highest drug concentration while avoiding toxicity, are defined for each drug. What differs with the use of TDM in infectious diseases is that the level of bacterial resistance affects the efficacy and might vary between different infected individuals. Therefore, the level of resistance of the bacteria should also be assessed.

Figure 6. Therapeutic drug monitoring (TDM) - taking drug exposure and bacterial level of resistance (minimum inhibitory concentration, MIC) into account to improve treatment outcome.

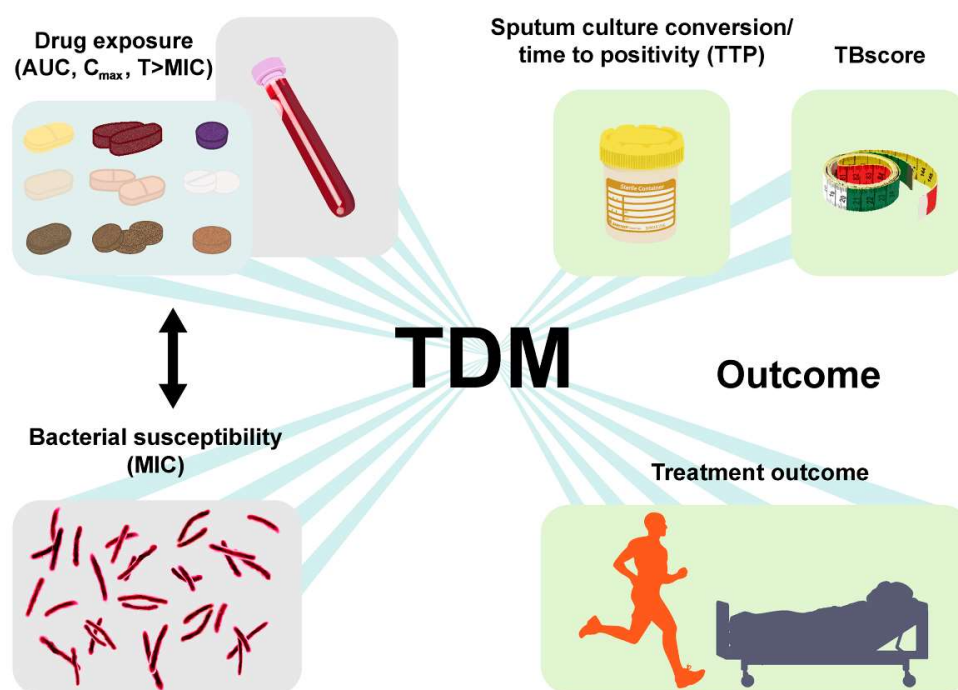


Illustration by Moi Peña 2018

2.6.1 Therapeutic drug monitoring in TB

Drug exposure of TB-drugs is greatly influenced by multiple factors such as concomitant food intake, comorbidities, malabsorption, drug-drug interactions and pharmacogenetics (104). Pharmacogenetics describes how genetics influences the response to a drug. For example, polymorphism genes encoding for metabolizing proteins will result in variable drug exposure between patients (101), for example isoniazid (101).

Low drug concentrations of TB drugs in blood in clinical settings have frequently been seen, although the majority of studies are from high-endemic areas (105). The drug exposure of recommended doses of first-line drugs in TB-patients in Sweden is unknown. Rifampicin is a particularly unpredictable concerning dose and drug exposure, due to variability in absorption as a result of genetic polymorphism as well as enhanced clearance after repeated doses owing to autoinduction of hepatic enzymes and increased pre-hepatic first-pass metabolism (106). Furthermore, the non-linear kinetics of rifampicin due to saturable biliary excretion and exposure-dependent bioavailability means a dose increase might result in a more than proportional increase in the drug exposure (107, 108). The recommended ranges of the first-line TB drugs are based on early pharmacokinetic studies in healthy volunteers and TB patients on treatment, often lacking correlation with treatment outcome (109, 110).

The plasma drug concentration might not reflect the concentrations at the site of infection, depending on the drug's pharmacodynamic characteristics. The recommendations above do not take clinical variables into account, such as concomitant HIV infection, the bacterial MIC or the site of infection. It should also be noted that a prerequisite for TDM is a clearly defined relationship between plasma concentrations and bacterial killing in TB patients, which has not yet been defined for all TB-drugs (111, 112). There is a need to define the optimal drug exposure, also in relation to the bacterial resistance, since the PK/PD target is unknown for many drugs. Despite knowledge gaps, TDM for TB is endorsed by many experts, including the American Society of Infectious Diseases and the European Respiratory Society and is used clinically in many settings.

In a systematic meta-analysis, there was no evidence to support the use of TDM as routine care for susceptible TB (105). Drug concentrations were frequently low, but only linked to treatment outcome in three out of 12 studies evaluating treatment outcome. However, there was heterogeneity in the number of blood samples collected and a relative uncertainty of the estimation of C_{max} and AUC. An overview of PK/PD studies including outcome measures regarding first- and second-line drugs are presented in Table 6.

2.6.2 New treatment strategies for MDR-TB needed

Despite progress and bold goals to combat TB, there remains substantial impoverishment, death and suffering caused by TB around the world. In summary, new tools for treatment improvement of TB and MDR-TB are urgently needed, such as new drugs or methods for personalized medicine in the field of TB.

Table 6. Clinical studies reporting drug exposure in relation to clinical outcome measure. A PubMed search with search-terms “Therapeutic drug monitoring” OR TDM OR PK/PK OR “Pharmacodynamics/pharmacokinetics” OR Concentrations) AND (Outcome OR Treatment OR Conversion OR Success OR Fail OR Failure) AND (Rifampicin OR Isoniazid OR Ethambutol OR Pyrazinamide) AND (Tuberculosis) was performed (July 2018) found 146 studies, of which 23 reported an outcome measure (bacteriological or treatment outcome) for adults with drug-susceptible TB. Studies comprising adult patients, with an aim to assess drug exposure or dose increase in association with an outcome measure (bacteriological or treatment outcome) for (RIF/R), isoniazid (INH/H), pyrazinamide (PZA/Z) and/or ethambutol (EMB/E) on daily treatment were included. Asterix (*) highlights studies where drug concentrations correlated to the outcome measure (11 out of 23 studies).

Author	Drug/doses	Study design	No. of patients	Sampling post dose	Exposure	Outcome measure	Results
Velasquez 2018 (106) Peru “HIGH RIF I”	RIF 10, 15 and 20 mg/kg for 8 weeks + HZE	RCT phase II	180 PTB (5 HIV+)	120 patients: 0, 2, 6 h 60 patients: 0, 0.5, 1, 1.5, 2, 6, 14 h	Dose mg/kg, AUC ₀₋₆ and AUC ₀₋₆ /MIC	Change in log ¹⁰ CFU and SCC at week 8.	No difference in SCC at week 8. Increased rate of CFU decline for the higher RIF dose. AUC and AUC/MIC not related to outcome.
Aarnoutse 2017 (107) Tanzania “HIGH RIF II”	RIF 600mg, 900 and 1200 mg + HZE	RCT phase II	63 TB (15 HIV+)	Eleven samples from 0-24 h at week 6	RIF AUC, C _{max} & dose	tSCC	No difference in bacteriological response; not powered for small differences
Alkabab 2017 (108) USA	Standard doses HRZE	Retrospective cohort, matched	363 PTB (pre/post-intervention, DM 56)	2 h at week 2	TDM RIF & INH as intervention (dose adjustment)	tSCC and SCC at month 2	*Significantly shorter tSCC for post-intervention group (-8 days) and -20 days for DM- compared to DM+ patients. Reduced tSCC at month 2 for post-intervention group
Boeree 2017 (109) Tanzania & South Africa	RIF 10 mg/kg, 20 mg/kg, 35 mg/kg +/- MFX + HZE	RCT phase 2 (SQ109 stopped)	365 PTB (24 HIV+)	NA	Increased dose without PK measured	tSCC in liquid and solid media	*tSCC was significantly quicker in 35 mg/kg vs control group (median 48 vs 62 days) but not in the other arms. No difference in tSCC on solid media.

Rockwood 2017 (110) South Africa	Standard doses HRZE	Prospective observational cohort study	100 PTB (65 HIV+)	0, 2, 3, 4, 6, 8h at week 8	AUC/MIC and C_{max} /MIC of RIF, INH, PZA	SCC and treatment outcome	*Low drug concentrations for RIF 80%, INH 43% and PZA 53%. PZA <35 mg/L predictive of failure/relapse. Concentration-dependent antagonism at low RIF and INH concentrations for SCC at month 2.
Heemskerk 2016 (111) Vietnam	RIF 15 mg/kg+LFX 20 mg/kg + standard HZE	RCT	TB meningitis (349 HIV+)	NA	Intensified treatment vs standard treatment	9 months: death and neurologic disability	No significant effect of the intensified treatment
Maze 2016 (112) New Zealand	Standard doses HRZE	Retrospective cohort study	121 TB	2 h	High vs low C_{2h} RIF & INH	tSCC and treatment outcome	Low RIF and INH concentrations (35 patients) were neither associated with successful treatment outcome nor tSCC. Dose adjustments might have contributed to the favourable outcome.
Boere 2015 (113) South Africa HIRIF I	RIF 10mg/kg vs RIF 15, 20, 25, 30 mg/kg for 2w+HZE	RCT phase II	68 PTB patients (1 HIV+)	Rich-sampling PK curve	PK of RIF	Fall in CFU and TTP in sputum samples	*There was a greater estimated fall in sputum bacterial load in the higher dosing groups
Chigutsa 2015 (114) South Africa	Standard doses (FDC) HRZE	Subset of a clinical trial	54 PTB (7 HIV+)	4-8 samples during 0-7 h	AUC, C_{max} , AUC/MIC, C_{max} /MIC, MIC	Fall in TTP and SCC at month 2.	*Low C_{max} for RIF (<8.2mg/L) and low AUC/MIC (<11.3mg*h/L) associated with slower bacterial elimination. RIF C_{max} <8.2 mg/L associated with a lower proportion of SCC at month 2
Mah 2015 (115) Canada	Standard doses HRZE	Retrospective cohort study	134 PTB (33 HIV +)	2, 6 h	C_{max} of RIF & INH	SCC month 2 and treatment outcome	*Low concentrations of INH associated with a positive sputum culture at month 2 (OR 3.3 95CI 1.33–8.33), similar but non-significant results for RIF. No association with the final treatment outcome.

Meloni 2015 (116) Switzerland	Standard doses HRZE	Retrospective cohort study	17 TB (7 HIV+)	2, 4, 6 h	C _{max} of RIF, INH, PZA, EMB & RIB	Treatment outcome	16 of 17 patients had at least one drug with low concentration and 94% were cured. Dose adjustment was performed.
Park 2015 (117) South Korea	Standard doses HRZE	Retrospective cohort study	413 TB	2 h	C _{2h} of RIF, INH, PZA, EMB	SCC 2m + treatment outcome	Low concentrations had no association with SCC or treatment outcome. Dose adjustment was performed for a >50%.
Prahl 2014 (118) Denmark	Standard doses HRZE	Prospective observational	32 TB (2 HIV+)	2 h	C _{2h} of RIF, INH, PZA, EMB	Treatment outcome (death/relapse)	*Low concentrations of INH (71%), RIF (58%), EMB (46%) and PZA (10%). Low drug concentrations of both RIF and INH associated with therapy failure (5 patients)
Burhan 2013 (119) Indonesia	Standard doses HRZE	Prospective cohort study	181 PTB (2 HIV+)	2 h	C _{2h} RIF, INH & PZA	SCC at month 2	91% had one drug (RIF, INH, PZA) lower than recommended. Drug concentrations were not associated with SCC at month 2.
Van Tongeren 2013 (120)	Standard doses HRZE	Retrospective cohort study	52 TB (12 HIV+)	2 h	C _{2h} RIF & INH	Acquired drug resistance	*Low drug concentrations frequent; INH (76.6%, associated with male sex) and RIF (68.4%). Five patients with low drug exposure showed acquired drug resistance. Dose adjustments were made.
Pasipanodya 2013 (121) South Africa	Standard doses HRZE	Prospective cohort study (severe cases)	142 PTB (15 HIV+)	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 h at month 2	C _{max} and AUC for RIF, INH & PZA	SCC and treatment outcome	*Significant association with treatment outcome; PZA AUC _{0-24h} ≤ 363mg*h/L, RIF AUC _{0-24h} ≤ 13mg*h/L & INH AUC _{0-24h} ≤ 52mg*h/L and with SCC month 2; PZA C _{max} ≤ 58.3, RIF C _{max} ≤ 6.6 and INH C _{max} ≤ 8.8
Ruslami 2013 (122) Indonesia	Standard RIF 10 mg/kg vs iv RIF 13 mg/kg + HZE	RCT phase 2	50 TB-meningitis	0, 1, 2, 4, 6 and 24 h at day 3	PK and C _{max} of RIF, EMB, PZA	Mortality 6 months after start	*Mortality reduction by 50% with intravenous increased dose of RIF, no added benefit of MFX.

Requena-Méndez A 2012 (123) Peru	Standard dose HRZE	Cross-sectional observational study	105 TB patients (26 DM+, 29 HIV)	2, 6 h	C _{max} of RIF	PK RIF, DM, HIV +/- & treatment outcome	No effect of DM or HIV on RIF concentrations. Out of five patients with poor treatment outcome, four had low drug exposure.
Chideay 2009 (124) Bostwana	Standard doses of HRZE	Prospective cohort study	225 PTB (155 HIV+)	1, 2, 6h	C _{max} and AUC of HRZE	Treatment outcome	*Low drug concentrations were frequent; (RIF 84%, INH 37%, EMB 39% and PZA 5%) and associated with HIV. Low PZA exposure associated with poor treatment outcome.
Chang 2008 (125) Hongkong	Standard doses HRZE	Case-control study	74 PTB	2, 4 h	C _{max} RIF	SCC at month 2	No association between RIF C _{max} and SCC. Controls were patients with SCC at month 2 and cases patients with no SCC at month 2
Ruslami 2007 (126) Indonesia	10 vs 13 mg/kg of RIF + HZE for 6 months	RCT phase II	50 PTB (1 HIV +)	0, 1, 1.5, 2.5, 3, 4, 6 and 12 h at week 6.	AUC and C _{max} of RIF	SCC and treatment outcome	No difference in SCC and treatment outcome, although not powered to assess.
Van Crevel 2002 (127) Indonesia	Standard doses HRZE	Prospective, observational	62 (1 HIV+)	2 h	C _{2h} of RIF	Treatment outcome	70% had very low (<4 mg/L) of C _{2h} , no difference in treatment outcome was seen. The strongest predictor of drug exposure was drug manufacturer (bioavailability)
Narita 2001 (128) USA	Standard doses HRZE	Retrospective, cohort study	188 (69 HIV+)	2 and 6 h at week 2	C _{max} RIF and INH	Recurrent TB	Recurrence of TB was not associated with low drug levels or HIV infection.

CFU: colony-forming units, DM: Diabetes Mellitus, E: ethambutol, FDC: Fixed dose combination, HIV: Human immunodeficiency virus, H: isoniazid, LFX: levofloxacin
MFX: moxifloxacin, NA: not applicable, p: patients, PTB: pulmonary TB, R: rifampicin RCT: Randomised controlled trial, standard doses: according to WHO
recommendations, tSCC: time to sputum culture conversion, TTP: time to positivity in liquid culture, Z: PZA

3 AIMS

3.1 OVERALL AIM OF THE THESIS

The aim of this thesis is to explore different approaches to combat MDR-TB, such as repurposing drugs, elucidating the importance of the level of drug resistance of TB-drugs as well as the role of therapeutic drug monitoring in the treatment of MDR-TB.

3.2 SPECIFIC AIMS OF THE INCLUDED STUDIES

- To determine the effect of TMP-SMX and MEM-CLA on susceptible and drug-resistant *M.tuberculosis* (**Study I&II**).
- To study the importance of individual mycobacterial MIC and its association with sputum culture conversion and treatment outcome in MDR-TB (**Study III**)
- To investigate individual drug exposure (C_{\max} & AUC) in drug-susceptible TB-patients and the corresponding mycobacterial MIC (**Study IV**)

4 MATERIAL AND METHODS

An overview of methods is presented below. More detailed information is found in the respective articles.

4.1 MATERIAL / STUDY DESIGN AND SETTINGS

4.1.1 Studies I & II

Unique *M. tuberculosis* isolates were studied, consisting mainly of MDR/XDR-TB isolates, including clinical isolates from patients from different continents, and isolates submitted to the supranational reference TB-laboratory at the Public Health Agency of Sweden for proficiency DST. The pan-susceptible strain H37Rv (ATCC 27294) was used as control.

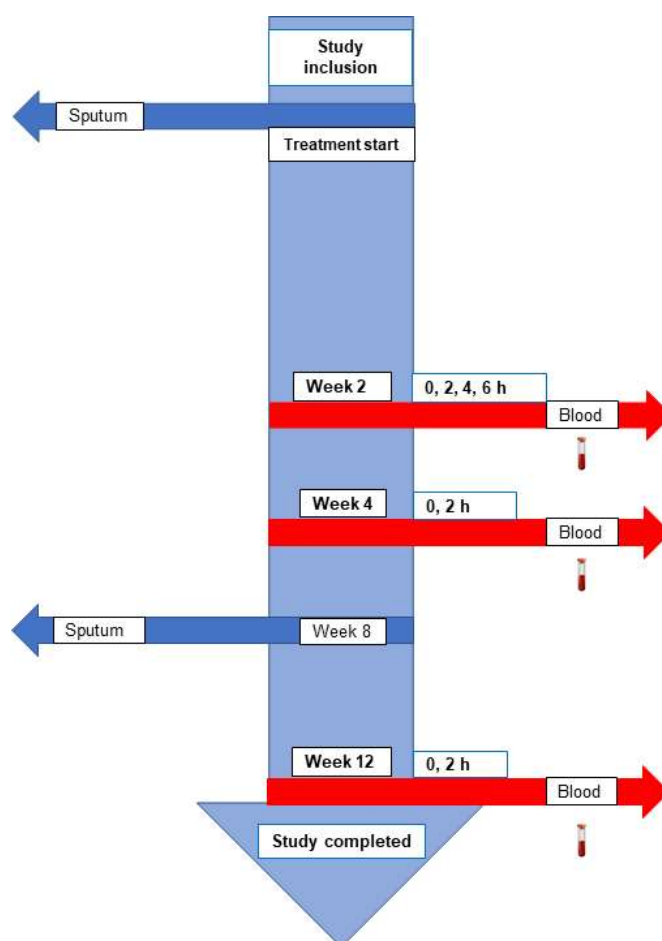
4.1.2 Study III

Due to mandatory reporting of TB, the Swedish national TB registry offers full-coverage of all diagnosed cases in Sweden. In this retrospective cohort study, all MDR-TB cases diagnosed in Sweden 1992-2014 were included (n=163). Five medical records had been lost, leaving 158 patients for analysis. Data were collected concerning sociodemographic information (i.e. gender, age, country of origin, years in Sweden, asylum status, use of interpreter), clinical characteristics (smoking, alcohol/drug use, height, weight, comorbidities, radiology reports, treatment regimens and duration, adverse drug reactions, tSCC, treatment outcome and microbiological data including routine DST). A treatment duration of one month or longer was defined as having received treatment with that drug. The MDR-TB diagnosis was phenotypically confirmed at the TB-laboratory of the Public Health Agency of Sweden, a national and supranational reference laboratory.

4.1.3 Study IV

In this prospective cohort study, patients with active, fully susceptible TB at Karolinska University Hospital Solna and Linköping University Hospital in Sweden were consecutively included from October 2012 until December 2014. Patients with MDR- or XDR-TB, ongoing infection other than HIV or unable to give informed consent, were excluded from the study. Plasma concentrations of all first-line drugs were measured after two weeks of TB treatment as well as after four and 12 weeks of treatment. The drug exposure was then compared to the individual mycobacterial MICs. The final treatment outcome was retrieved from the national TB registry and compared to the medical records.

Figure 7. Overview of study design of Study IV.



4.2 METHODS OF DRUG SUSCEPTIBILITY TESTING (DST)

The proportion method is the reference method for DST for *M.tuberculosis*. The growth of a standardised bacterial inoculum on a drug-free medium is compared with the growth on medium containing the critical concentration of a drug. A 1% critical proportion is used to define resistance to TB-drugs (apart from 10% for pyrazinamide) (40). Potential sources of error include bacterial inoculum standardisation, drug-containing suspension, media pH, incubation time and temperature as well as the validity of the critical concentration (129).

4.2.1 Solid LJ media – Study III

Solid media using LJ was used for DST testing of cycloserine and PAS in **Study III**. Bacterial suspensions, calibrated to McFarland 0.5, are inoculated in drug-containing LJ media. A diluted bacterial suspension (1/100) is added to a growth-control slant incubated at 37°C. Resistance is defined as growth in the drug-containing slant exceeding the growth in the control tube, with at least 30-100 visible colonies. Growth is evaluated at week four, where resistance is evident, and at week six for susceptible isolates (63). The drug-containing media is often prepared locally, with risk of variable quality.

4.2.2 BACTEC 460 TB and BACTEC MGIT 960 – Study III & IV

The older BACTEC 460 TB used radioactively-labelled tubes and measured CO₂ production, whereas BACTEC MGIT 960 detects oxygen consumption due to metabolic activity. Fluorescent material is embedded at the bottom of the MGIT tubes and the consumption of oxygen releases fluorescence which can be detected. Pre-manufactured MGIT tubes containing the critical concentration for the first-line drugs are commercially available, increasing the quality of the result but also costs. The tubes are incubated in BACTEC MGIT 960 until a level of 400 Grown Unit (GU) for the control tube is detected and the tubes are read. The growth of the tube containing the critical concentration of the drugs is compared to the growth of the 1:100 diluted drug-free control tube. More growth (>100 GU) at a critical concentration compared to the 1:100 diluted control is regarded as resistance (130).

Table 7. Critical concentrations used for first-line drugs and important second-line drugs in BACTEC MGIT 960 in Study III.

Drug	mg/L
Rifampicin	1
Isoniazid	0.1, 0.4
Pyrazinamide	100
Ethambutol	5
Ofloxacin	2
Amikacin	1

4.2.3 Drug susceptibility testing of pyrazinamide - Study III & IV

Susceptibility testing of pyrazinamide is pH-dependent (28) and liquid broth with a low pH of 6.9 is used. However, the low pH itself inhibits the growth of *M.tuberculosis* isolates (28), which is why a bacterial suspension of 1:10, instead of the traditional 1:100, is used as growth control according to manufacturer instruction. The most common source of error in pyrazinamide DST is the inoculum size, where an excessively large inoculum will increase the pH and might lead to false resistance (64). Measures to decrease the risk are to sonicate the inoculum to homogenize the solution and to use fresh *M.tuberculosis* isolates, no older than two weeks (131). The McFarland turbidity is performed manually which is a potential source of error.

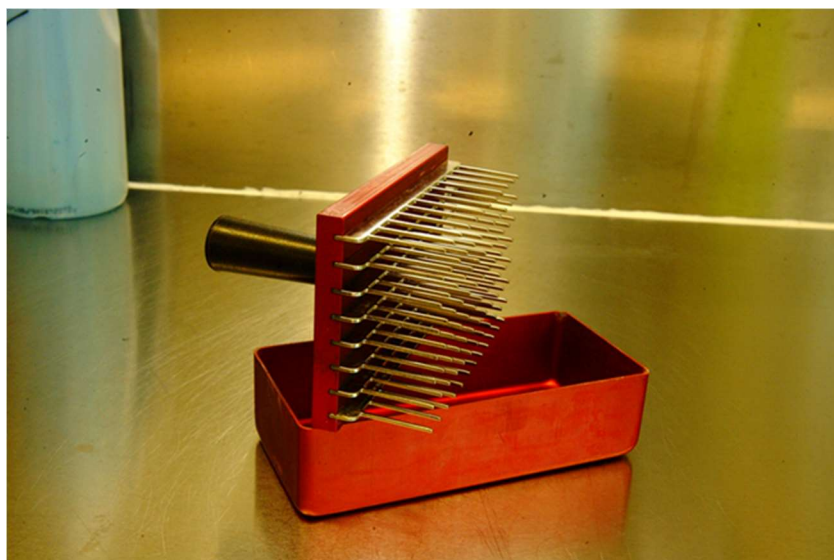
4.3 MINIMAL INHIBITORY CONCENTRATIONS – SOLID, LIQUID OR MICROTITRE METHODS

4.3.1 Solid media Middlebrook 7H10 - Study I & II

Enriched Middlebrook 7H10 agar (Becton Dickinson AB, Stockholm, Sweden) was casted into 14-cm petri dishes. Serially diluted stock solutions of TMP-SMP and MEM-CLA were added (final ranges 0.008-8/0.15-152 TMP-SMX and 0.002-512 MEM with CLA 64 mg/L

added uniformly). A 96-stick replicator (Figure 8) was used to transfer bacterial inoculums to the plates, in order to enable simultaneous testing of a large number of isolates. The pan-susceptible *M.tuberculosis* strain H37Rv was used as a control in all dishes and the MIC was defined as the lowest concentration with less growth than the 1:100 diluted control, thereby representing > 99% inhibition.

Figure 8. A 96-stick replicator used to inoculate agar plates.



4.3.2 Intracellular versus extracellular MIC testing – Study I

An H37Rv strain carrying the gene *Vibrio harveyi* luciferase was used and incorporated in a macrophage model for determining intracellular MIC. Luminescence is emitted by *Vibrio harveyi* luciferase only in the presence of living bacteria and the intracellular growth of *M.tuberculosis* was estimated by recording luminescence.

4.3.3 MIC testing using BACTEC MGIT 960 - Study I, III & IV

In MGIT tubes, serial 2-fold dilutions of antibiotics were added and incubated in BACTEC MGIT 960 according to the manufacturer's instruction. The MIC was defined as the lowest antibiotic concentration with less bacterial growth compared with the 1:100 diluted controls (for pyrazinamide 1:10 diluted controls).

4.3.4 Microtitre Sensititre™ plates – Study III & IV

The microtitre plates were used containing lyophilized freeze-dried antibiotics at increasing concentrations, enabling the simultaneous determination of 14 antibiotics. Standardised bacterial inoculums were added to the wells, incubated at 37°C and read after 10-21 days, assisted by an inverted mirror. The H37Rv strain was included as an internal control in all plates. The MIC was defined as the lowest concentration able to inhibit visible growth. The sensitivity of detecting resistance of drugs compared to BACTEC MGIT 960 is still variable, over 95% for rifampicin but 72% for ethambutol (MYCOTB plate) (68).

4.4 WHOLE GENOME SEQUENCING (WGS)

WGS provides a comprehensive analysis of the genome (132) and was used in **Study III** for relapse confirmation. DNA is extracted from an *M.tuberculosis* isolate, sequenced and the result of the sequencing is compared with a reference sequence in order to detect single nucleotide polymorphisms (SNPs), i.e. variations in a single nucleotide, insertions or deletions (133). A true relapse was defined as less genetic distance than 6 SNPs difference between the paired isolates (134). WGS requires pure, double-stranded DNA to perform well and cannot be used on direct sputum samples, as currently.

Principle steps of WGS

1. Culture of *M.tb* isolate (LJ/MGIT)
2. DNA extraction
3. Whole genome sequencing
4. Analysis of sequence data

4.5 DRUG CONCENTRATION MEASUREMENTS

Liquid-chromatography mass-spectrometry (LC-MS) was used to determine the total plasma drug concentrations in **Study IV**. The free fraction of the drug was calculated using previously published values of protein binding in humans. In LC, liquid solvents are passed through a column containing a solid adsorbent, with strong affinity for hydrophobic molecules, and thereby the compounds are separated depending on their degree of interaction with the adsorbent material. The chromatographically separated compounds are then detected by mass spectrometry. Mass spectrometry measures the mass-to-charge ratio of charged particles that are separated by electromagnetic fields and can quantify the amount of a compound in a sample (135). Internal standards are vital to account for day-to-day variability of the method.

4.6 EPIDEMIOLOGY

4.6.1 Cohort study design

Cohort study design was used to enable investigation of the association between exposure and outcome and to follow participants over time, up to the outcome of interest or censoring (death, lost to follow up etc) (**Study III & IV**). Multiple outcomes can be studied and time to event data is created, allowing for survival analysis, suitable for relatively common outcomes (136).

4.6.2 Random error

Random error is unpredictable variation by chance and can be reduced by an increase in the number of observations. The confidence interval (CI) is a range in between two values and the level of confidence (p-value) tells us the probability that the true value lies within this range. At a significance level of <0.05 , as in our studies, the CI would likely include the true value in 95 out of 100 repeated studies (137).

4.6.3 Bias

Apart from random error, different forms of bias (potentially leading to incorrect conclusions) are divided into three main categories; selection bias, information bias and confounding.

Selection bias occurs from systematic differences in characteristics between the study participants and the population from which they are selected; for instance, oversampling healthier patients able to come for study enrolment (137). In clinical studies screening logs are kept to assess the risk of selection bias. Selection bias was minimized in **Study III** since 97% (158/163) of all patients were included in the analysis.

Information bias refers to the correctness of the data, i.e. errors in the classification of exposure and outcome. Prospectively collected data avoids the misclassification of exposures depending on the outcome. Missing data might be a form of information bias, if the missingness is differential. Non-differential missing data, i.e. missing by chance, might only dilute the result.

Confounding bias is a variable linked both with the exposure and the outcome and unevenly distributed between the two comparison groups, resulting in a distortion of the association. An example is a study of hospitalised patients showing a protective effect of obesity and risk of death, when this might be explained by the presence of a serious medical illness, resulting in both low BMI and high risk of death. Residual confounding can be reduced, but perhaps not eliminated, in the analysis stage by statistical adjustment for possible confounders (136).

4.7 STATISTICAL CONSIDERATIONS

In general, a p-value <0.05 was considered significant for all studies and all tests were two-sided. Normal distribution of continuous variables was assessed using histograms. For comparison of categorical data, Pearsons' chi-square test or Fisher's exact test (for small sample sizes) were used.

The time to event data in **Study III** was analysed using Cox proportional hazard regression models. Cox regression estimates the risk of experiencing an outcome, a so called hazard function, comparable to a speed. Unlike Poisson regression, the individual hazard rate cannot be estimated, only the hazard rate ratio between two different groups, concurrently adjusted for the effect of other variables, often confounders in a multivariable regression model. The proportional hazard assumption for Cox models always has to be tested, since the underlining assumption is that the hazard rates are proportional over time, which can be tested using Schoenfeld residuals, for example. Cox proportional hazard models automatically adjust for the time scale, in this case since start of MDR-TB treatment and no assumptions regarding the distribution are needed (138). The Cox regression model was chosen in **Study III** for the unique availability of time to event data and the view that the time to poor outcome is of interest, not just the final outcome. In a country with a high proportion of successful treatment outcome, the time to event is of interest to discern differences between groups of patients. The non-parametric Kaplan-Meier estimator visualises the probability of staying outcome free over time and was used to visualize differences in time to sputum culture conversion in **Study III**. The Kaplan-Meier estimator can be used for censored data, with the assumption that censoring is non-informative censoring (138).

4.8 ETHICAL CONSIDERATIONS

Medical ethics relies on the principles of non-maleficence, justice, beneficence and autonomy (139). The axiom “*Primum non nocere*”, implores the medical profession to, above all, not do our patients harm (non-maleficence). Health care should be fair and equal (justice). In medical research the well-being of the patient or the study participant should be the primary goal (beneficence), through the means of a thorough cost-benefit analysis regarding potential risks. Patient autonomy, which emphasises the right of the patient to make independent decisions, is a term closely linked to informed consent.

Studies I & II. Both studies involved growth and enrichment of MDR/XDR-*M.tuberculosis* isolates, exposing the staff to an increased bio-hazard. However, the laboratory work was performed at the TB laboratory at Karolinska University Hospital by experienced staff, minimising this risk. No ethical approval was needed.

Study III. A strength of this retrospective cohort study is the completeness of the data, with all MDR-TB patients in Sweden consecutively included over a 22-year period. This was enabled through a waiving of an informed consent, a potential breach of patients’ integrity. The need of patient approval would have likely resulted in a marked decrease in patient inclusion, especially since many patients had returned to their country of origin and some had died. Efforts were made to minimise this breach of integrity by the use of only three medical staff bound by confidentiality in performing data collection. The knowledge gained from the study is deemed to exceed the potential harm to the study participants. Approved by the Regional Ethic Board of Linköping (Dnr: 2012/197-31).

Study IV. Repeated blood samples were the main cause of patient discomfort in the study. Three blood samples were collected after two weeks of treatment, when most patients were out-patients. More blood samples would have made PK estimates more accurate, but in the interest of patient comfort and time, a minimal number of samples were chosen.

The use of an informed consent in order to respect patients’ autonomy should ensure that the patient makes an independent, non-coerced, knowledgeable decision. The patient might feel obliged to participate in order to please the doctor, which is why specially trained study nurses informed and included the patients. Patients were also informed, orally and in writing, that they were free to leave the study, without providing any reason, with no effect on their future treatment. The study was performed according to Good Clinical Practice and the Helsinki Declaration.

Finally, one can argue from a deontological, or duty-based perspective, that the harm from blood sampling goes against our duty as doctors of non-maleficence. From a utilitarian perspective, on the other hand, the benefit to society is also taken into account, i.e. the potential usefulness of the study for other patients (139). The Swedish study has revealed unexpectedly low drug exposure during standard treatment, important knowledge for clinicians treating TB. In conclusion, the anticipated benefits of the studies are considered to outweigh the risks. Approved by the Regional Ethic Board of Linköping (Dnr: 2012/197-31).

5 RESULTS

5.1 STUDY I

Trimethoprim-sulfamethoxazole has in vitro activity against highly resistant M.tuberculosis isolates, at clinically achievable doses.

The 84 drug-resistant strains tested for TMP-SMX in 7H10 solid medium showed an MIC distribution ranging from 2.4 to 38 mg/l of SMX (0.125/2.4 to 2/38 mg/l of TMP-SMX) (Figure 9).

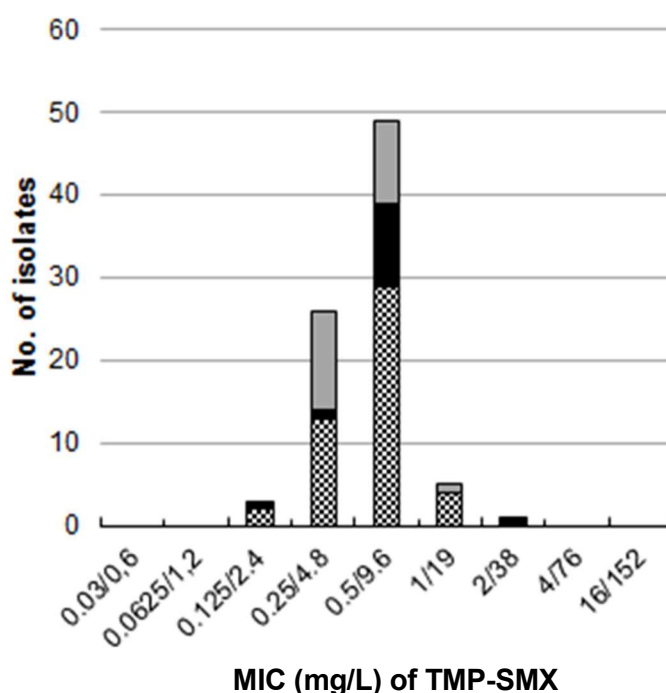


Figure 9. Effects of trimethoprim-sulfamethoxazole (TMP-SMX) on *M. tuberculosis*. MIC distribution of TMP-SMX for the *M. tuberculosis* isolates (n=84) tested using Middlebrook 7H10 medium, shown by bars shaded as follows: non-MDR/XDR-TB (grey), XDR-TB (black), and MDR-TB (hatched).

In another experiment using liquid 7H9 medium, TMP showed no effect on its own, or in combination with TMP-SMX, which is why the results of the active component SMX are reported. The extracellular effect of SMX was 19 mg/L.

The intracellular growth was evaluated using a macrophage model where H37Rv-lux was phagocytized by macrophages and emitted luminescence was measured. The intracellular MIC₉₀ of SMX was 76 mg/L. Even at higher levels of SMX (120 mg/L) some viable bacilli were seen, indicating low intracellular penetration of the drug. The extra-and intracellular effect was evaluated in triplicates with an experimental method using 96-well plates (Sarstedt) with Middlebrook 7H9 broth.

We also performed an exploratory analysis of potential targets for the $fAUC_{0-24}/MIC$ ratio (25, 50, and 75) for SMX. The probability of target attainment and cumulative fraction response values for different doses up to 7,200 mg of SMX was estimated using Monte Carlo simulations (105). A cumulative fraction response of 90%, including MDR- and XDR- *M.tuberculosis* isolates, was reached at doses of 2,400 mg, 3,600 mg, and 7,200 mg of SMX using target indices of 25, 50, and 75, respectively (Figure 10). If a PK/PD target of 25 is used, as suggested for melioidosis and for *M.tuberculosis* (140, 141), one tablet of Bactrim® forte (co-trimoxazole) thrice daily (2400 mg daily) is sufficient to kill over 90% of the MDR/XDR-TB isolates.

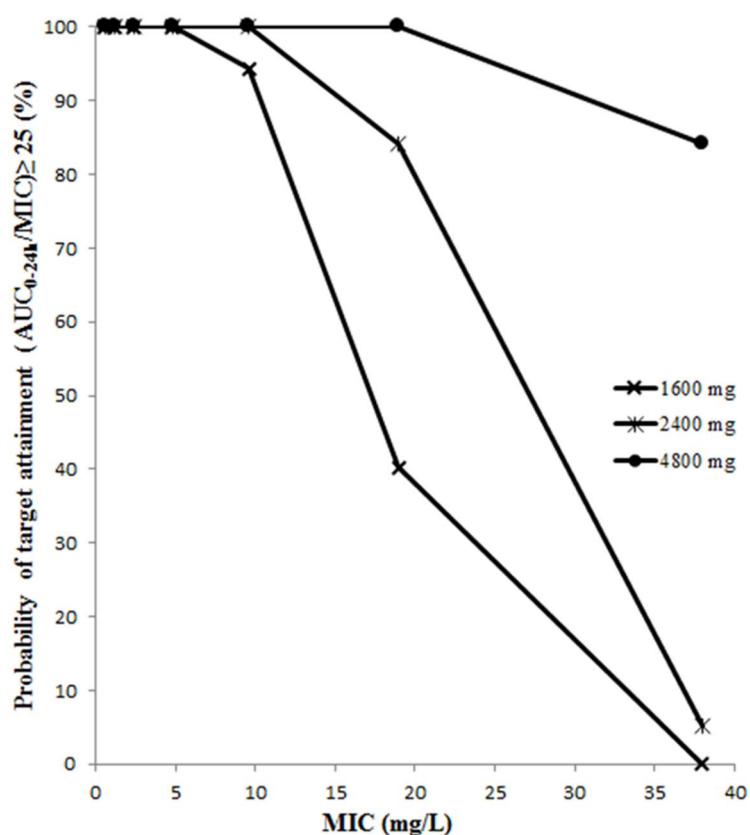


Figure 10. Probability of target attainment (PTA) after selected different daily sulfamethoxazole doses and a $fAUC_{0-24h}/MIC$ ratios of 25.

5.2 STUDY II

Meropenem-clavulanate acid is effective against MDR/XDR-TB isolates in vitro

We investigated the activity of MEM-CLA against 68 *M. tuberculosis* isolates. We included predominantly MDR/XDR-TB isolates. Using Middlebrook 7H10 medium, all but four isolates showed an MIC distribution of 0.125 to 2 mg/L for MEM-CLA, below the non-species-related breakpoint for MEM of 2 mg/L defined by EUCAST, and pertaining to a 1g thrice daily dosing regimen.

The range of the MIC distribution was 0.125-2 mg/L for MEM in combination with CLA (Figure 11). Most of the MICs were normally distributed following a wild-type pattern below the non-species-related breakpoint for MEM of 2 mg/L. However, four isolates had high MICs (16 and 32 mg/L respectively) and were deemed resistant. The resistant isolates also showed extensive resistance to other structurally unrelated anti-TB drugs, suggestive of poor drug permeability as a possible resistance mechanism. The MIC of the control strain H37Rv was 1 mg/L.

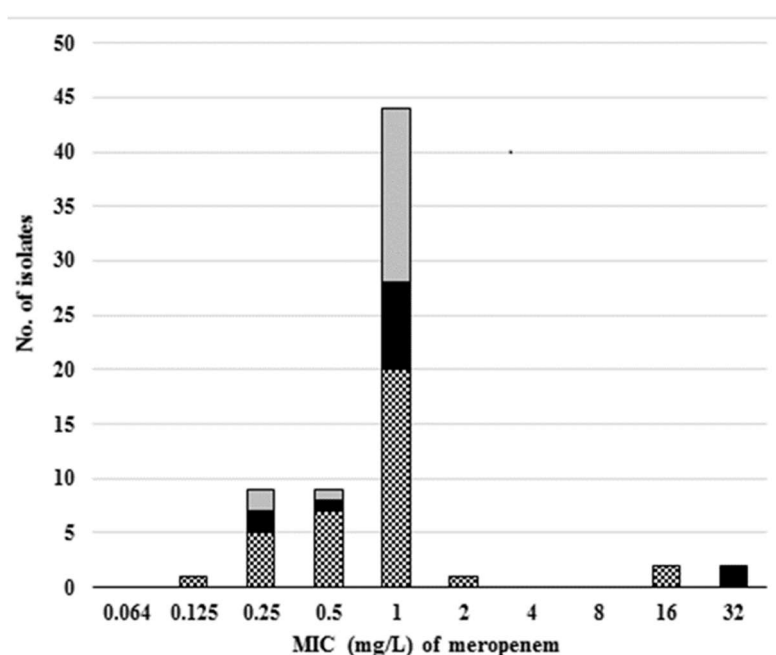


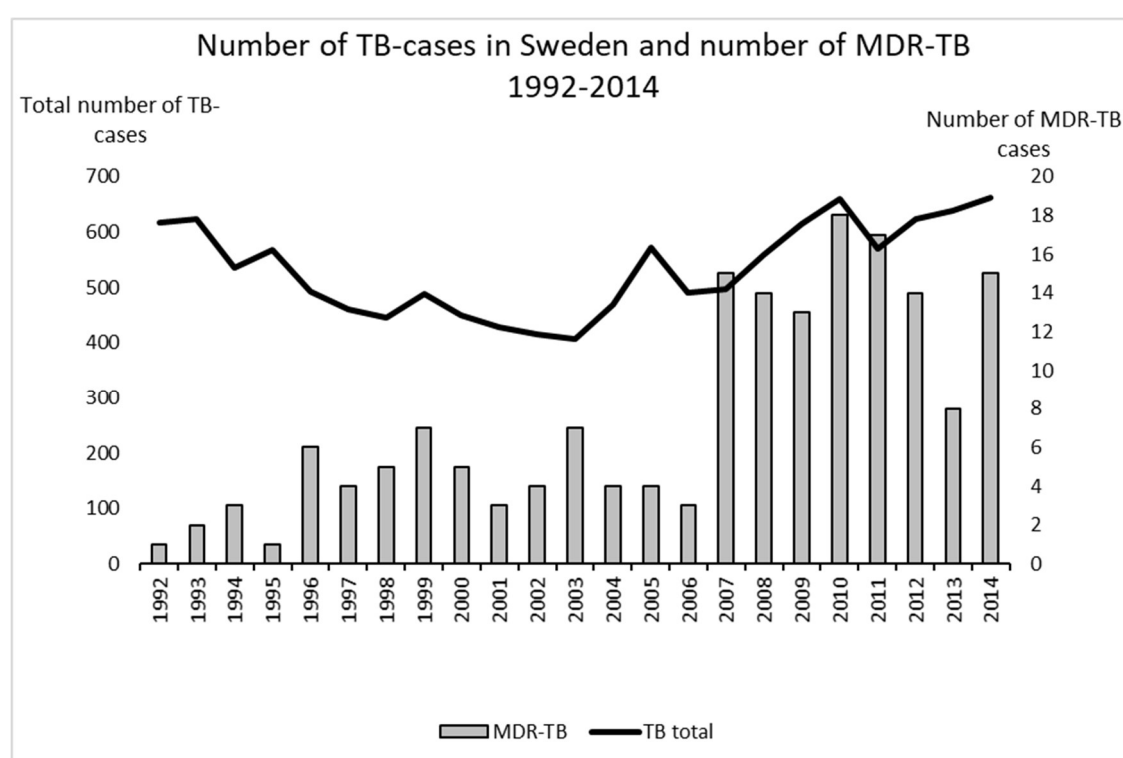
Figure 11. The MIC distribution of meropenem-clavulanic acid for the *M. tuberculosis* isolates (n=68) tested using Middlebrook 7H10 medium, shown by bars shaded as follows: non-MDR/XDR-TB (grey); XDR-TB (black), and MDR-TB (hatched). The MIC distribution shows the concentration of meropenem, as the concentration of clavulanic acid was fixed uniformly at 64 mg/L.

5.3 STUDY III

Minimum inhibitory concentrations of fluoroquinolones and pyrazinamide susceptibility correlate to clinical improvement in MDR-TB patients

There were 163 patients diagnosed with MDR-TB in Sweden from 1992-2014, with a marked increase in the incidence of MDR-TB during this time period, as shown in Figure 12. The median age was 29 (range 1-85 years) and 42.4% were female (67/158). The vast majority of patients were born outside of Sweden (94.3%; 149/158). Comorbidities were infrequent, the most common being HIV (10 patients) and diabetes mellitus type 2 (seven patients). PTB was the most common form of TB (122; 77.2%), of which the majority were sputum smear positive (65/118, 55.1%).

Figure 12. The trend in TB and MDR-TB in Sweden.



Resistance to important first-and second line drugs was 52.5% for pyrazinamide, 43.7% for ethambutol, 15.2% for any second-line injectable drug (amikacin, kanamycin or capreomycin) and 10.1% for ofloxacin. Patients previously treated for TB (first/second-line treatment) were more likely to harbour an *M.tuberculosis* isolate resistant to fluoroquinolones (22.2% vs 5.5% for untreated patients; $p=0.002$) and second-line injectables (24.4% vs 11.8% respectively; $p=0.049$), but not resistant to ethambutol or pyrazinamide.

The most common backbone treatment was ethambutol, pyrazinamide, levofloxacin and amikacin, in combination with either prothionamide, cycloserine or linezolid, in order of decreasing frequency. There were 48 (30.3%) patients eligible for the WHO MDR-TB short course regiment, although it was not in use or recommended during the study period.

MIC determination was possible for 142 of 158 *M.tuberculosis* isolates (10 isolates not stored, 6 with no growth). An increase in MICs of levofloxacin, ofloxacin and ethionamide

was significantly associated with an increased risk of poor treatment outcome (composite outcome of death/failure/relapse and death/failure/relapse/lost to follow-up) (Table 8). In a complete case analysis, the result was still significant after adjustment for confounders such as age, gender and year of diagnosis. However, for patients treated with a fluoroquinolone and with a susceptible isolate, there was no significant association for levofloxacin in the multivariate model (aHR 1.70 95% CI 0.85-3.40 p=0.134) although the association remained significant for ofloxacin (death/failure/relapse/lost to follow-up, aHR 1.25 95% CI 1.02-1.53 p=0.030)

Other variables associated with treatment outcome were diabetes and age of diagnosis, with a 4.5 times increased rate of death/failure/relapse/lost to follow-up for patients older than 40 years (aHR 4.51 95% CI 1.74-11.67 p=0.002).

Table 8. Univariate analysis regarding MICs of first- and second line TB drugs and composite treatment outcome (death/failure/relapse and death/failure/relapse/lost to follow-up). The analysis was restricted to patients with a susceptible isolate who received treatment with the drug or group of drugs analysed. For example, for analysis of levofloxacin, all patients treated with a fluoroquinolone with a levofloxacin/ofloxacin susceptible isolate were included.

		Death/Failure/Relapse nr= 15/158	Death/Failure/Relapse/Lost to follow-up nr=22/158		
MIC mg/L	Total	HR (95% CI)	p-value	HR (95% CI)	p-value
Group A					
Levofloxacin	125	2.08 (1.10-3.92)	0.024	2.00 (1.19-3.37)	0.009
Moxifloxacin	125	2.09 (0.30-14.52)	0.458	2.06 (0.42-10.07)	0.374
Ofloxacin	125	1.33 (1.03-1.73)	0.030	1.43 (1.19-1.72)	<0.001
Group B					
Amikacin	118	0.98 (0.77-1.24)	0.852	0.98 (0.80-1.18)	0.799
Kanamycin	118	1.01 (0.92-1.10)	0.899	1.03 (0.98-1.09)	0.265
Capreomycin	115	1.06 (0.94-1.19)	0.326	1.08 (1.01-1.16)	0.025
Group C					
Prothionamide	77	1.11 (0.79-1.56)	0.555	1.17 (0.92-1.49)	0.205
Ethionamide	77	1.25 (1.05-1.48)	0.013	1.17 (1.01-1.37)	0.038
Cycloserine	85	0.95 (0.82-1.11)	0.522	0.96 (0.86-1.07)	0.459
Linezolid	62	0.89 (0.00- 2141.01)	0.977	5.79 (0.02-1716.30)	0.545
Group D					
Ethambutol	74	0.88 (0.65-1.21)	0.436	0.84 (0.62-1.16)	0.289
PAS	38	0.06 (0.00-55.57)	0.426	1.11 (1.03-1.19)	0.009
Clinical data					
Gender	154	1.08 (0.34-3.42)	0.893	1.33 (0.52-3.39)	0.548
Age	154	1.07 (1.04-1.10)	<0.001	1.05 (1.03-1.08)	<0.001
Year of diagnosis	154	0.93 (0.86-1.02)	0.109	0.96 (0.89-1.03)	0.248
Diabetes	152	8.97 (1.87-43.1)	0.006	8.09 (2.30-28.5)	<0.001

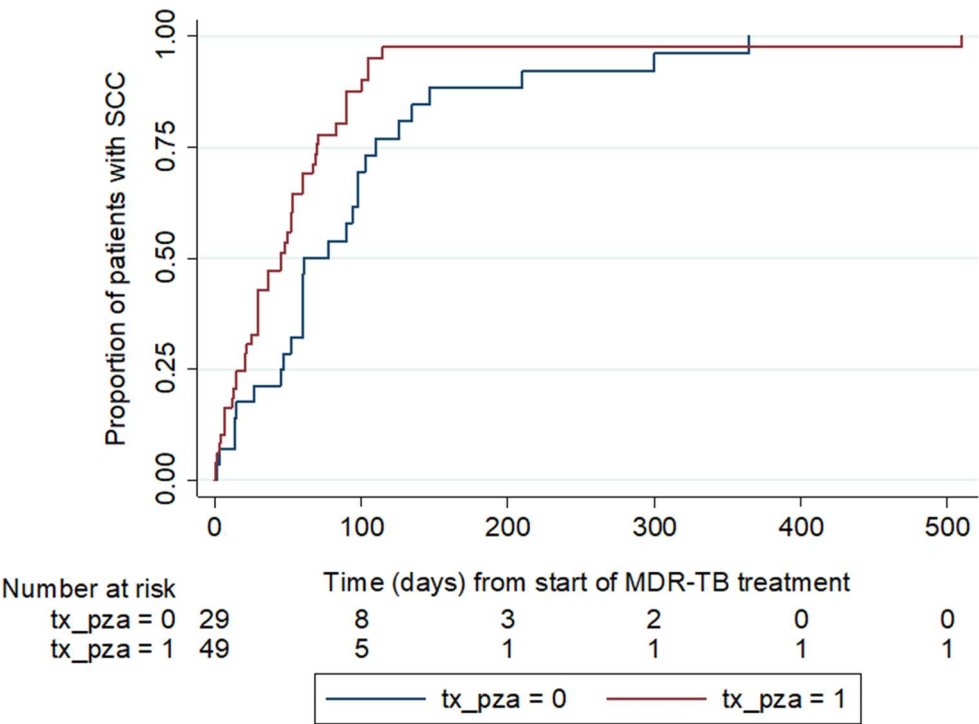
For all drugs above, the analysis was restricted to patients with a susceptible isolate who received treatment with the drug or group of drugs analysed. For example, for analysis of levofloxacin, all patients treated with a fluoroquinolone with an ofloxacin susceptible isolate were included. No patients were treated with bedaquiline or delamanid and no MIC determination of pyrazinamide was performed. Analysis of MIC of clofazimine not possible due to too few treated patients. PAS= para-aminosalicylic acid. Diabetes mellitus type II, no cases of type I. Non-significant variables: gender, alcohol, smoking, previous TB treatment, HIV, Body Mass Index (BMI), cavitory pulmonary TB, pre-XDR/XDR-TB, number of resistant drugs.

There were 122 PTB patients and sputum culture conversion was achieved for 84 of the 87 patients with available information of time to sputum culture conversion. The median time to conversion was 46 days (IQR 13.8-79.3). After adjustment for potential confounders such as gender, age, year of diagnosis, previous TB and smear positivity, variables such as previous TB treatment, smear positivity at diagnosis and pyrazinamide resistance were significantly associated with an increased time to sputum culture conversion.

On the other hand, pyrazinamide treatment was not significantly associated with time to culture conversion after adjustment of confounders in a complete case analysis. Almost a third of patients (31.7%, 26/83) received treatment with pyrazinamide despite pyrazinamide resistance, in most cases due to a delay in the DST results. Thus, when stratifying on pyrazinamide treatment and resistance, PZA treatment for patients with PZA susceptible Mtb isolates was significantly associated with a reduced tSCC of one month (aHR 2.25 95% CI 1.27-3.99 p=0.005).

However, there was no discernible effect on sputum culture conversion for patients receiving pyrazinamide treatment with a resistant isolate. The difference in time to sputum culture conversion depending on pyrazinamide treatment is visualised in a Kaplan-Meier curve Figure 13.

Figure 13. Kaplan-Meier failure-curve for time to sputum culture conversion from start of MDR-TB treatment and treatment of pyrazinamide (tx_pyrazinamide=1, red line).



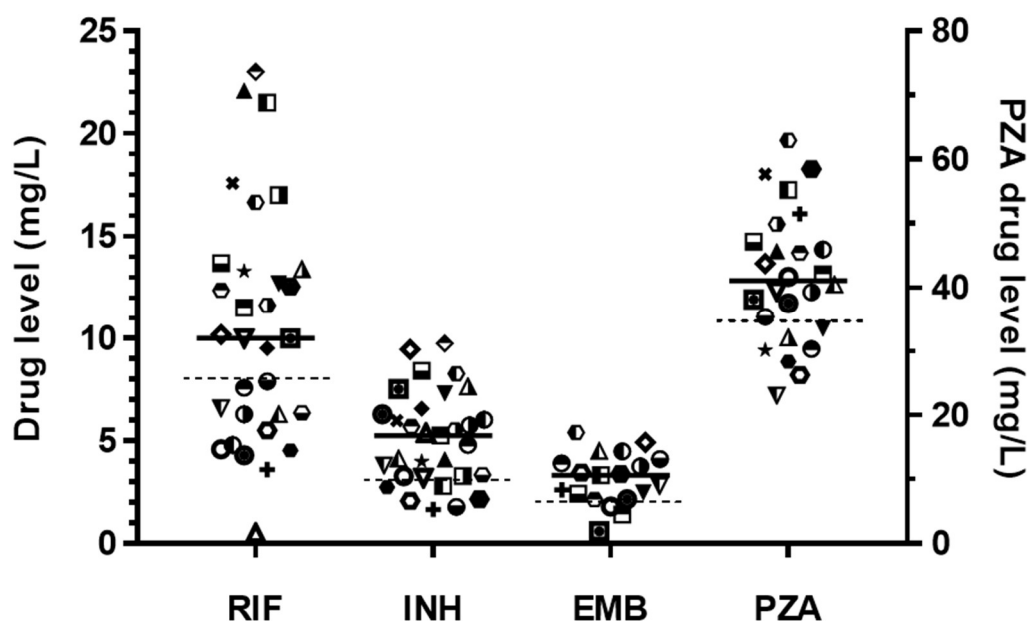
5.4 STUDY IV

Low drug concentrations of first-line drugs are commonly low in TB-patients

There were 31 consenting adult TB-patients included in the study. The median age was 33 years (IQR: 26-44) and the majority of patients were female and originated from sub-Saharan Africa (61%, 19/31). Comorbidities according to the Charlson comorbidity index (142) were infrequent (3/31, 10%) and there were no HIV co-infected patients. Twenty-six patients had bacteriologically confirmed TB and among pulmonary TB patients, 21% (5/24) had cavitory disease and 25% (6/24) were sputum smear positive. All patients were initially deemed cured at the time of treatment completion, but two patients experienced recurrence of TB.

After two weeks of treatment, lower than recommended drug concentrations were detected in 42 % of the patients for rifampicin (<8 mg/L), 19 % for isoniazid (<3 mg/L), 16% for ethambutol (<2 mg/L) and 27% for pyrazinamide (<35 mg/L). The median C_{high} for rifampicin was 10.0 mg/L (range 0.5-23.0 mg/L), for isoniazid 5.3 mg/L (range 1.6-9.8 mg/L), for ethambutol 3.3 mg/L (range 0.6-5.4) and for pyrazinamide 41.1 mg/L (range 23.1-63.0) for pyrazinamide. The distribution of plasma levels of C_{high} for each of the four first-line drugs is displayed in Figure 14.

Figure 14. Distribution of plasma levels of C_{high} for first-line TB drugs at week 2 in patients with active TB.



Footnote: Individual total drug levels of rifampicin, isoniazid, ethambutol and pyrazinamide are plotted against the left y-axis whereas the right y-axis corresponds to the pyrazinamide plasma level. For each individual patient the same dot style is used throughout for all drugs. Horizontal bold lines represent median levels and horizontal dotted lines show the lower recommended limit for each drug according to the literature (RIF 8 mg/L, INH 3 mg/L, PZA 35 mg/L and EMB 2 mg/L) (119) (110). RIF=rifampicin INH=isoniazid EMB=ethambutol PZA=pyrazinamide.

Individual rifampicin concentrations displayed significantly more fluctuation over time compared to isoniazid concentrations ($p < 0.001$). The median C_{high} of isoniazid was markedly lower for fast acetylators compared with slow acetylators (3.2 vs 6.2 mg/L, $p = 0.002$), at all measured time points (weeks 2, 4 and 12).

In MGIT, the median MIC levels for susceptible isolates were 0.064 mg/L (range 0.016-0.125) for rifampicin, 0.032 mg/L (0.032-0.064) for isoniazid, 1 mg (1-4 mg/L) for ethambutol and 32 mg/L (16-100 mg/L) for pyrazinamide. None of the isolates exhibited any known drug resistance mutations by the WGS-analysis for the first-line drugs.

When comparing the drug exposure with the individual MICs, median $C_{\text{high}}/\text{MIC}$ for rifampicin, isoniazid, ethambutol and pyrazinamide were 164, 128, 2.5 and 1.3 respectively whereas median $\text{AUC}_{0-6\text{h}}/\text{MIC}$ was 636 (range 156-2759) for rifampicin and 351 (range 72-895) for isoniazid. The corresponding calculated $fC_{\text{high}}/\text{MIC}$ and $f\text{AUC}_{0-6\text{h}}/\text{MIC}$ for rifampicin were 33 (range 3-157) and 128 (range 31-554) respectively.

6 DISCUSSION

6.1 MAJOR FINDINGS

We found high *in vitro* effect of TMP-SMX and MEM-CLA, even against *M.tuberculosis* isolates with resistance to multiple other TB-drugs (MDR/XDR-TB) (**Study I&II**). In our retrospective cohort study, the overall treatment outcome in MDR-TB patients in Sweden 1992-2014 (no=158) was favourable with 86% successful treatment outcome and seven deaths. Variables associated with unsuccessful treatment outcome (death/failure/relapse) were increments of MIC of fluoroquinolones as well as age and diabetes. The appropriate use of pyrazinamide in the treatment for MDR-TB was associated with an approximate reduction in time to sputum culture conversion of a month (**Study III**). Finally, drug concentrations were commonly lower than recommended for susceptible TB patients, despite standard treatment dosages (**Study IV**).

6.2 GENERAL DISCUSSION

Clinicians pose themselves many questions regarding how to best treat their patients – what is the best choice of drugs, ideal dose and optimal treatment duration for this particular individual? In TB-patients, we want to ensure a rapid halt of contagiousness, tolerable treatment regimens and a durable cure, without acquired drug resistance. In order to do this, we need to consider the intricate relationship between host (pharmacogenetics, immune status), pathogen (bacterial resistance and virulence) and drug exposure (PK characteristics).

6.2.1 The quest for new drug regimens – in with the new, in with the old

The repurposing of drugs by finding new applications for already approved drugs can reduce time to approval of new drug alternatives. We studied the *in vitro* effect on MDR-*M.tuberculosis* isolates of the readily available TMP-SMX (Bactrim®/co-trimoxazole) (**Study I**). Our tentative breakpoint of 2/38 mg TMP-SMX has been endorsed by others (143, 144). The results have also recently been corroborated in a study of 153 MDR-*M.tuberculosis* isolates where the MIC distribution was <0.05/1 – 1.7/32 mg/L of TMP-SMX, although performed using a colorimetric method (resazurin microdilution in 7H9). No sign of resistance was seen for patients previously treated with TMP-SMX (n=51) (144). Moreover, in a small PK study where patients received 480 mg of SMX daily, only one out of eight patients had a $fAUC_{0-24h}/MIC$ ratio of SMX greater than 25, a previously suggested PK/PD target (145). Our exploration of potential targets for $fAUC_{0-24h}/MIC$ with Monte Carlo Simulations showed that an adequate cumulative fraction response (CFR > 90%) was achieved at doses of 2400 mg of SMX daily, corresponding to a clinically tolerable dose of three tablets of Bactrim® forte daily. It should be emphasized that the PK/PD target of 25 is an expert opinion extrapolated from PK/PD targets for melioidosis (caused by the gram-negative bacteria *Burkholderia pseudomallei*), but not validated clinically for TB.

There is limited clinical data regarding TMP-SMX as large studies, as well as PK/PD dose-ranging studies, are lacking. The active component of the combination, SMX, has poor lipid solubility and exhibits poor intra-cellular activity, limiting its use to complicated MDR-TB cases during the early phase of disease, where bacteria are found extracellularly (146).

Another repurposed drug, MEM-CLA, is recommended as a Group C drug against MDR-TB (77). Carbapenems are intrinsically a poor substrate for the beta-lactamase of *M.tuberculosis* (89) and a synergistic effect with clavulanic acid has been shown (147). **Study II** is in line with previously reported results where MEM-CLA displayed high bactericidal activity even against drug-resistant *M.tuberculosis* isolates, with MICs predominantly below 2 mg/L (89).

A small clinical randomised controlled trial (RCT) (n=30) showed a significant reduction in bacterial load (1.5 decline of log CFU count) when MEM-CLA (2 g MEM and 500/125 mg of amoxicillin-clavulanic acid thrice daily) was added to standard treatment of susceptible TB for two weeks (148). There is however limited clinical data and the short half-life of one hour requires frequent intravenous dosing, limiting its use. Ertapenem is a newer carbapenem with a longer half-life, allowing for once daily administration. Five cases of pre-XDR/XDR-TB patients treated with ertapenem in combination with WHO recommended MDR-TB treatment, resulted in a successful outcome for four patients (149). In a retrospective study including 180 patients, Tiberi and co-authors suggested that MEM-CLA acid is more effective than imipenem-clavulanic acid in the treatment of MDR/XDR-TB. However, it should be noted that there were significantly more patients with fluoroquinolone-resistance in the imipenem group (79% vs 48.9%, n= 84) and since no multivariable analysis was performed (150) there is a risk of confounding.

Since the *in vitro* activity of meropenem without a beta-lactamase inhibitor is relatively poor (MIC levels up to 32 mg/L) (151), meropenem is only recommended when given together with amoxicillin-clavulanic acid (77). New beta-lactamase inhibitors include avibactam and vaborbactam, and have been combined with beta-lactam antibiotics (i.e. zavicefta®; ceftazidim-avibactam and vabomere®; meropenem-vaborbactam) (152, 153). The drawback of only intravenous availability has been overcome by tebipenem, the first orally available carbapenem, approved in Japan for treating children with drug-resistant pneumococci. Tebipenem inhibits the beta-lactamase of *M.tuberculosis* and might be worth exploring further, clinically (154). Carbapenems are a treatment option in very difficult to treat patients and are generally well tolerated (155), although the long-term effect of a changed microbiota has not been evaluated for TB patients.

The drug bedaquiline, introduced clinically in 2013, has very recently been given the highest recommendation for MDR-TB treatment and its use is increasing world-wide. The drastically changed recommendations include an upgrading of linezolid and cycloserine, at the expense of second-line injectables.

Not only are the recommended treatment regimens changing, the way we view resistance and DST is currently under scrutiny.

6.2.2 Reliable drug susceptibility and MIC-testing do matter

DST of *M.tuberculosis* should be performed but it needs to be reliable and reproducible to ensure appropriate treatment choices. Access to DST will also avoid patients experiencing adverse events due to ineffective drugs. Increased mortality has been seen for patients with discordant DST results (156). In a multi-centre study from TB high-burden countries, 634 TB patients were included, of which 193 patients (30.4%) had MDR/XDR-TB. Comparing DST results from a local laboratory with the reference laboratory, there were 121 discordant results

and consequently 27 patients (22.3%) were assessed as having received inadequate treatment regimens according to WHO guidelines, compared to only eight (1.8%) patients with concordant DST results. Similarly, there were also marked differences in mortality between the two groups (17% mortality with concordant DST versus 30.8% with discordant DST $p=0.030$) (156).

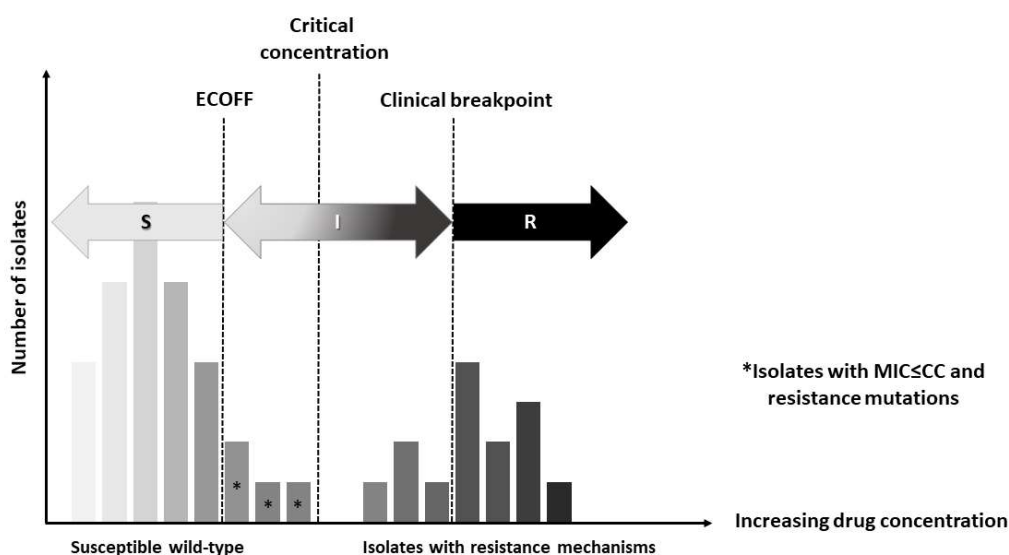
In **Study III**, the decreased time to sputum culture conversion was only seen among patients treated with pyrazinamide and diagnosed with a pyrazinamide-susceptible *M.tuberculosis* isolate, whereas no reduction was seen in patients treated with pyrazinamide with a pyrazinamide-resistant *M.tuberculosis* isolate. In the largest meta-analysis of MDR-TB so far, the use of pyrazinamide was also associated with slightly lower mortality (adjusted risk difference 0.03), but only if the isolates were susceptible for pyrazinamide (78). These findings highlight the need of a reliable DST for pyrazinamide to identify individuals who may benefit from pyrazinamide treatment. Unfortunately, pyrazinamide DST is rarely performed in many high-burden settings. Unavailable or inaccurate DST results might lead to standardised or inaccurate treatments, stressing the need for a reliable DST for all second-line drugs.

6.2.2.1 *Resistance is continuous, not binary*

For the DST to be of clinical value, the critical concentration has to be relevant and validated, which unfortunately is often not the case. In **Study III**, there was no correlation between treatment outcome and DST results with current critical concentrations. Admittedly, critical concentrations were difficult to evaluate in a small study like ours, especially given that several drugs were given. In the aforementioned large individual patient data meta-analysis by Ahmad and co-authors, drug resistance was associated with worse treatment outcome (78). However, our study exemplifies the difficulty in setting relevant critical concentrations and highlighting the continuous nature of resistance. Increments of MIC for levofloxacin and ofloxacin were, however, associated with unsuccessful treatment outcome, including relapse. Previous studies have shown higher MICs of isoniazid and rifampicin, although still below the critical concentrations, to be predictive of poor treatment outcome (157). Similarly, a recent study showed an association between higher baseline MIC for either rifampicin or isoniazid and an increased risk of relapse, even if all *M.tuberculosis* isolates were susceptible according to current critical concentrations (158). In this case-control study, *M.tuberculosis* isolates from 54 relapse TB patients were included from a previously performed study and 63 cured TB patients were randomly selected from the same cohort. In a predictive model based on MICs and clinical characteristics, the MIC level of rifampicin or isoniazid, lack of sputum culture conversion after two months of TB-treatment as well as being underweight showed independent association with increased risk of relapse. The authors of this study concluded that the level of resistance of the *M.tuberculosis* isolate is at least as important as clinical characteristics such as underweight and cavitary TB, previously identified as a risk factor for TB relapse (158). As previously stated, relapse of TB is thought to be caused by the inability to clear persisting bacteria which often hide in a necrotising caseum where drug penetrations may vary (159). It is possible that it is the combination of a sub-population of *M.tuberculosis* together with an increased MIC residing in difficult to treat locations that makes eradication more difficult. An MIC-based approach to select patients for shorter treatment durations with less risk of relapse has therefore been suggested (158).

Since binary breakpoints are difficult to define and have the inherent risk of oversimplifying resistance, the testing of two breakpoints has been suggested, similar to that of isoniazid (0.1 and 0.4 mg/L) (160). An intermediate-susceptible dose-dependent category for TB has been proposed, similar to other bacteria, where resistance can be overcome by increased dosage (160) but this is not widely accepted. For example, intermediate categories, or even more appropriate “*susceptible, increased exposure*” as defined by EUCAST, have been suggested for isoniazid, pyrazinamide and rifampicin (161, 162). A clinical breakpoint in MGIT of moxifloxacin of 1.0 mg/L, compared to a critical concentration of 0.25 mg/L, when increased dosage of 800 mg daily is used, has recently been recommended by WHO (40).

Figure 15. *M. tuberculosis* isolates with increased resistance compared to ECOFF and/or the critical concentration that might still be treated with increased dosing, belonging to an intermediate category, or “*susceptible, increased exposure*” (I).



6.2.2.2 MIC values – advantages as well as pitfalls

In contrast to DST results, MIC determination allows for a more nuanced understanding of the level of resistance. MIC determinations in MGIT are costly, time-consuming and labour-intensive. Microtitre plates allow for the simultaneous MIC determination of 14 different drugs, at a low cost with results within two weeks, making MIC determinations more accessible in clinical practice (67). However, MIC methods, similar to other methods, are prone to some variability. A reported MIC value should not be viewed as the “true” value, but as providing information whether the isolate is of wild-type or not (66). In other words, an MIC value should be viewed as predictive of probability of cure on a continuous scale rather than an exact value.

With these caveats in mind, MIC values might still guide clinicians regarding the degree of resistance to a drug, in order to deduce what drug exposure might be needed. Knowledge of the drug exposure in our patients is also needed. TDM is a useful tool to elucidate the relationship between bacterial resistance and individual drug exposure.

6.2.3 Therapeutic drug monitoring – a torch in the dark

6.2.3.1 Low drug exposure common in TB-treatment

Low drug concentrations are common in TB-treatment, even in a high-resource setting with high quality drugs, using recommended dosing in an otherwise healthy cohort (**Study IV**). In a meta-analysis, the frequency of low drug concentrations at 2 hours (C_{2h}) for first line drugs was shown to be 67% for rifampicin, 43% for isoniazid, 27% for pyrazinamide and 12% for ethambutol. Subtherapeutic levels of rifampicin and isoniazid were more frequently seen in the meta-analysis than in **Study IV** (rifampicin 67% vs 42%, isoniazid 42% vs 19%), possibly since the meta-analysis mainly included studies from high-burden countries and not all studies with strict fasting conditions as ours (163). However, AUC calculation is a more precise measure of drug exposure, since C_{2h} might be low due to delayed absorption rather than low drug exposure. The median AUC_{0-6} of rifampicin in **Study IV** was 35, marginally lower than the mean AUC at steady state of 38.73 mg·h/L from a recent meta-analysis, displaying the representativeness of the data (164).

Interestingly, patients in **Study IV** with low rifampicin concentrations (<8 mg/L) had significantly higher TBscore II, possibly explained by altered PK characteristics in severely ill patients. Indeed, reduced absorption of rifampicin has been seen in critically ill patients (165) whereas the volume of distribution of lipophilic drugs such as rifampicin is not as affected (27). An additional contributing factor is that observed reduced drug concentrations after a few weeks of rifampicin treatment are not only due to autoinduction of metabolizing hepatic enzymes, but also decreased oral bioavailability (93% to 68% after three weeks' treatment) due to the induction of pre-hepatic metabolism (166).

The median C_{high} in **Study IV** of pyrazinamide was 41.1 mg/L, lower than the identified cut-off by Pasipanodya where a C_{max} below 58.3 mg/L was associated with poor two-month sputum culture conversion (121). In **Study IV**, all but one patient had sputum culture converted by month 2, despite the majority having lower C_{high} than 42 mg/L. Our study did not have sufficient power to assess differences in microbiological response.

When comparing the drug exposure in our cohort with the individual MICs, the PK/PD ratio was adequate, even for patients with very low drug concentrations, due to the low MIC range in the study (rifampicin range 0.016-0.125 mg/L). The median AUC/MIC of rifampicin was 636, significantly higher than the suggested target value of 271 (167). It should be noted that the tentative PK/PD target for rifampicin was derived from a murine aerosol infection model with dose fractioning over six days and has not been validated in human beings. Two previous studies with Monte Carlo simulations have reported low probabilities of target attainment of rifampicin $AUC/MIC \geq 271$ at 600 mg daily (168, 169), illustrating that adjustment of the target might be needed. However, PK/PD targets derived from murine models use monotherapy and does not take the synergistic effect of drug into account (169).

The fact that none of the patients had a severely impaired immune system or HIV, might also have contributed to the favourable outcome, although the only two patients with relapse had low levels of rifampicin during the treatment course. Studies show that rifampicin appears to accumulate intracellularly with an alveolar macrophage drug concentration versus a plasma drug concentration of around 16:1 (170), but it is unknown whether this ratio is static and if

plasma concentrations always reflect intracellular drug concentrations. It should be pointed out that **Study IV** was not sufficiently powered to study differences in mortality or final treatment outcome between patients with low or adequate drug exposure.

6.2.3.2 *Does low drug exposure matter?*

As shown in Table 6 in the Introduction section herein, there is conflicting evidence whether subtherapeutic drug concentrations are linked with poor clinical outcome, with 11 out of 23 studies showing an association. In a Danish study in a similar setting to ours, treatment failure (five out of 32 patients) was more common in patients with lower than normal C_{2h} drug concentrations of isoniazid and/or rifampicin (118). In a South African study, the three most important predictors of a poor clinical outcome were identified as pyrazinamide $AUC_{0-24h} \leq 363$ mg·h/L, rifampicin $AUC_{0-24h} \leq 13$ mg·h/L and isoniazid $AUC_{0-24h} \leq 52$ mg·h/L (121). In a meta-analysis up to 2014, only three out of 12 studies assessing outcome (culture conversion or treatment outcome), showed an association between subtherapeutic levels and unsuccessful treatment outcome (163). Possible reasons for the conflicting results from the studies are differences in outcome definitions, study populations, PK sampling, drug formulations, HIV co-morbidity as well as too low power and too subtle variations in drug exposure, to have a clinical impact (110).

Fast acetylators of isoniazid (homogenous for the wild-type gene N-acetyltransferase 2 (NAT2)) had significantly lower drug exposure at all measured time points in our study (**Study IV**). Acetylator status of isoniazid varies across populations, where slow-acetylator status is related to an agriculture/postural life-style and more common in Scandinavia, as compared to in Japan where 53.5% of patients in a pharmacogenetic RCT of isoniazid were of the fast-acetylator genotype (171). In the aforementioned RCT, the dose of isoniazid was adjusted according to NAT2 genotype, rather than plasma concentrations, and reduced toxicity as well as improved clinical progress were seen (171), illustrating the possibility of pharmacogenetic-based therapy.

6.2.3.3 *Drug exposure in MDR-TB patients – a lesser studied field*

Less is known of drug exposure in MDR-TB patients, where treatment entails more drugs combined, leading to a substantial risk of suboptimal drug concentrations. Therefore, we performed a prospective clinical cohort study in Xiamen, China, regarding drug concentrations in relation to individual MICs in MDR-TB patients. We have included 30 patients and performed a richer sampling protocol for a better estimation of AUC as well as MIC and WGS determination (see published study protocol **Appendix 1**). Similarly to **Study IV**, we used markers of treatment progress such as TTP, TB-score, treatment outcome and a quality of life evaluation. The results are currently being analysed and will hopefully shed more light regarding drug concentrations in MDR-TB patients.

Adequate drug exposure is also important in preventing the development of drug resistance and low drug concentrations have been associated with acquired drug resistance in clinical studies (120, 121, 172). In a multi-centre study in high-endemic countries, up to 46% of MDR-TB patients with resistance to a second-line injectable developed XDR-TB during treatment (173). Indeed, previous authors have highlighted the importance of

pharmacokinetic variability leading to subtherapeutic drug exposure as a cause of resistance, through hollow-fibre experiments (174).

Since low drug concentrations are common and will potentially impair cure and lead to drug resistance, increased dose recommendations are being looked into.

6.2.3.4 Dose recommendations under revision

There is now supporting evidence that an increased rifampicin dose leads to increased drug exposure, without increased toxicity (107). In an important murine experiment from 2003, there was a clear relationship between exposure to rifampicin (AUC/MIC) and reduced bacterial burden in lung tissue (CFU) (167). Indeed, out of four randomized Phase II trials, all apart from one (107) showed that increased rifampicin exposure was associated with a more rapid clinical improvement of pulmonary TB (106, 109, 126). There was no effect on the final treatment outcome, although the studies were not powered to assess this outcome.

In a two-week long study, doses up to 35 mg/L were well tolerated and increased bactericidal activity was seen in the higher dosing groups (HIGHRIFF 1) (113). However, there were only 15 patients in each dosing group and the study duration was short. In the follow-up study HIGHRIFF 2 with an extended study period of two months, daily doses of rifampicin 900 and 1,200 mg resulted in an increased drug exposure but no reduction of time to sputum culture conversion, compared to standard treatment (107). In a three-month long study with 365 patients (PanACEA MAMS-TB-01), the median time to sputum culture conversion in liquid media only (but not on solid) was reduced two weeks for patients treated with 35 mg/kg compared to the standard dose of 10 mg/kg of rifampicin, without significant increase in toxicity (109).

Although mainly explored for pulmonary TB, increased dosing to improve treatment outcome for TB infections with high mortality, such as TB meningitis, has been studied. Despite the promising results of reduced mortality with 13 mg/kg intravenous rifampicin for TB meningitis (122), a phase 2 study with 817 patients did not show any benefit of an increased oral dose of rifampicin (15 mg/kg) (175), although the dose increase might not have been high enough.

To gain more knowledge from clinical studies regarding increased rifampicin dose, pharmacometric modelling studies have been performed. In a modelling study with data from the PanACEA MAMS-TB-01 trial, the proportion of patients with sputum culture conversion at week 8 increased linearly from 39% with 10 mg/kg to 55% with 35 mg/kg of rifampicin (176). Another modelling study based on the same data has shown similar results, where higher pyrazinamide exposure was associated with shorter time to sputum culture conversion (177). A dose-ranging clinical trial of levofloxacin from 11 mg/kg up to 20 mg/kg for MDR-TB is ongoing (Opti-Q NCT01918397).

6.2.3.5 Reducing treatment duration for susceptible TB

The finding of shortened time to sputum culture conversion by higher rifampicin doses led to exploration of a possible reduction in treatment duration for TB, so far only by pharmacokinetic modelling studies (176). However, despite convincing pre-clinical data (mice data/hollow-fibre) four clinical trials have failed to show support for a shortened four

months therapy through the addition of a fluoroquinolone, due to unacceptably high relapse rates of 10-20% (71-74). Rifampicin reduces the drug exposure of moxifloxacin around 30% (178), which was not taken into consideration as the moxifloxacin dose was not increased. Furthermore, it has been suggested that the insufficient sterilising activity of moxifloxacin has contributed to increased relapses, since imaging experiments on human lung resections have shown low drug concentrations in the caseum of cavities where persisters reside (159). This exemplifies that plasma drug concentration is a mere proxy for the drug concentration at the site of infection. Colangeli and co-authors recently argued that MIC determinations could be an aid in selecting patients with low MICs, where a shorter treatment duration might be appropriate (158), although this needs to be evaluated in clinical studies.

The efforts to reduce treatment duration of TB continue and the ongoing RIFASHORT trial is exploring a possible treatment duration of four months with rifampicin 1200 mg or 1800 mg (NCT02581527), as well as the NC005 trial with four months of treatment of bedaquiline, pretomanid, moxifloxacin and pyrazinamide NCT02193776. Furthermore, the long half-life of rifapentine as a way to reduce treatment duration is being studied (NCT02410772).

6.2.3.6 *Reduced treatment duration of MDR-TB*

Owing to its sterilizing effect, pyrazinamide is worth exploring in the quest to shorten MDR-TB treatment. The addition of pyrazinamide allowed the reduction of treatment of susceptible TB from 9 to 6 months (179). Pyrazinamide is currently included in several trials exploring new treatment regimens for MDR-TB (STAND, STREAM II, endTB) (86). The optimal dose and treatment duration of pyrazinamide should be evaluated, as result of pharmacokinetic modelling of clinical data suggest higher doses are needed (180). A synergistic effect of pyrazinamide with rifampicin, pretomanid (181) as well as bedaquiline has been seen in murine TB (182). Perhaps pyrazinamide might deserve a higher recommendation than currently suggested by WHO, where DST is available.

Clofazimine is another sterilizing drug with activity against hypoxic, non-replicating *M.tuberculosis in vitro* (183). In an RCT with 55 patients in China, the addition of clofazimine in the treatment of MDR-TB led to earlier sputum culture conversion and increased successful treatment outcome (53.8% compared to 73.6%) (184).

There are several ongoing clinical studies with new or repurposed drugs exploring the optimal regimen and treatment duration of MDR-TB, such as the NExT study (bedaquiline, linezolid), STREAM II (bedaquiline), endTB (bedaquiline +/-delamanid) and TB-PRACTECAL (bedaquiline, pretomanid). The STAND trial is evaluating bedaquiline, pretomanid and moxifloxacin for 6 months compared to standard MDR-TB treatment, where bedaquiline was added in 2016 to the initial study regimen (NCT03338621), owing to its high efficacy and sterilizing properties.

A more effective, shorter TB-treatment with affordable oral drugs in low-resource settings is a key priority. Since there is large variation in disease severity, patient characteristics and level of bacterial resistance, perhaps treatment durations need to be individualised more.

6.2.4 One size does not fit all – the need to individualise therapy

Individualised treatment takes multiple factors of a particular individual into account, such as clinical, microbiological and pharmacological aspects. Standardised regimens, without taking adverse drug reactions and DST results into account, might lead to increased toxicity and poorer outcome. Clinical characteristics, such as site of infection and immune status, are important to consider, although individual PK characteristics should not be ignored. For example, an increased ocular toxicity has been seen in obese patients with weight-based dosage of ethambutol. Since ethambutol does not easily distribute in fat-tissue, it should be dosed according to lean body weight to avoid overdosing (185).

Individualised therapy means more than just individualised dosing. If the rifampicin dose is doubled due to low drug concentrations, the drug exposure might be more than doubled, due to the non-linear kinetics of the drug (107, 126). Therefore, the actual drug concentrations need to be measured.

6.2.4.1 Individualised therapy through therapeutic drug monitoring

Given the prevalence of subtherapeutic drug concentrations in TB-patients, the possibility of TDM in clinical practice is being investigated. Figure 15 visualises a potential scenario of how TDM can be implemented clinically.

Figure 15. Illustration of TDM for tuberculosis treatment in clinical practice.

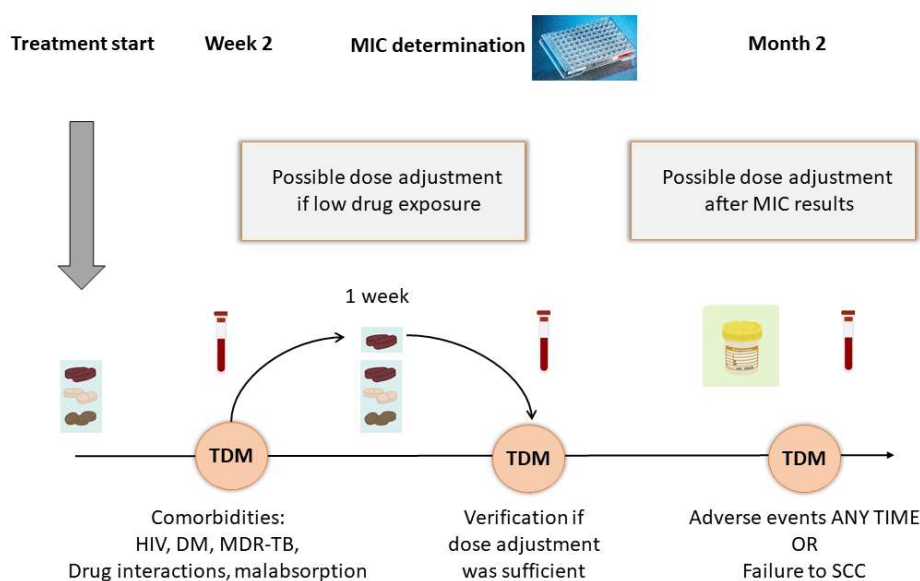


Illustration Lina Davies Forsman

Blood for measurements of drug concentrations is collected using limited sampling, when the drug is at steady state, typically after two weeks of treatment. Drug concentrations are then measured primarily for immunocompromised patients and/or patients at risk of low drug concentrations, such as patients with comorbidities (HIV, DM), malabsorption, severe disease as well as MDR-TB patients. Drug concentrations are then compared to reference ranges and, if available, the individual MIC. Valid PK/PD targets should be developed for the most important TB-drugs.

Importantly, the MIC value should not be regarded as an outright number, rather a range of plausibility of clinical success. An individual MIC should be inflated by at least one two-fold dilution or more, depending on the proficiency of the laboratory (186). The site of the disease also needs to be taken into account, where extensive cavitary disease or TB meningitis may require increased drug exposure. Dose adjustment can then be done if needed and a follow-up TDM is performed one or two weeks later.

As of today, there are no randomized trials evaluating the benefit of TDM for TB and it is not likely to be cost-effective for indiscriminate use. Patient groups with the greatest potential gain in the use of TDM should be identified; these are most likely to be immunocompromised patients with different comorbidities, including diabetes, as well as patients with severe infections such as TB-meningitis and disseminated TB. Furthermore, suggested PK/PD indices are derived from pre-clinical data and murine models and validation in TB patients is needed for valid PK/PD targets. Encouragingly, one of the primary aims of the Opti-Q trial is to determine the optimal tolerable AUC/MIC of levofloxacin. TDM at present is a useful clinical tool for treatment optimization primarily for immunocompromised and difficult to treat TB-patients, patients with slow clinical improvement and patients with MDR-TB, to ensure cost-effectiveness. However, the cost of acquired drug resistance or treatment failure is very high, both for the patient and society.

6.2.4.2 Concluding remarks

In this thesis, individualising TB treatment by taking bacterial resistance, drug exposure and disease and patient characteristics into account has been thoroughly discussed. However, any clinician knows that there is more to it. We also need to individualise patient care with regard to psychological factors such as fear, motivation and need of support. Recently, a Swedish mother of three described her MDR-TB treatment as the biggest trauma of her life (187). Future research needs to make sure that the treatment is not traumatic.

In the bigger picture, universal health coverage and addressing social determinants of TB are fundamental to curb the TB epidemic (33), especially since the highest burden of TB is in low-income countries. Advances in research and new tools must be made universally accessible, as well as possible to implement clinically. Multidisciplinary care involving clinical pharmacologists and microbiologists, infectious disease physicians, pulmonologists, surgeons and counsellors is of great importance. The most significant obstacle against individualised therapy is the increased need for surveillance and trained personnel, an extra burden on an already stretched health care system. These inequalities must be addressed, for no one should have to experience forced isolation away from their family, the stigma of having spread TB to your loved ones, the loss of a child to TB or debilitating and sometimes permanent side-effects. All TB patients around the world have the same right to optimal care, regardless of country of origin and wealth.

7 CLINICAL IMPLICATIONS AND CONCLUSIONS

- TMP-SMX and MEM-CLA showed good *in vitro* activity for MDR/XDR-*M.tuberculosis* isolates. Pharmacokinetic modelling indicates that TMP-SMX (i.e. Bactrim® forte /co-trimoxazole) can be used at clinically tolerable doses and may be considered as an alternative in difficult-to-treat drug-resistant TB.
- MEM-CLA also displayed high *in vitro* activity against resistant *M.tuberculosis* isolates and meropenem is already recommended by WHO as an add-on drug, in combination with the beta-lactamase inhibitor CLA.
- Our results indicate reduced time to sputum culture conversion by the appropriate use of PZA. PZA should be added to MDR-TB regimens if DST results indicate susceptibility.
- MIC determination might be a useful tool in quantifying resistance and the probability of successful treatment outcome of MDR-TB, especially for fluoroquinolones.
- Lower than recommended drug concentrations of first-line TB drugs are common, especially with more severe disease.
- The highest proportion of patients with low drug concentrations was seen for rifampicin, with significantly higher individual fluctuations compared to other first-line drugs.
- TDM is an important tool to unveil subtherapeutic drug exposure, especially important in severely ill or immunocompromised patients. Both variability in drug exposure, as well as the level of MIC, should be considered.

8 FUTURE PERSPECTIVES

Speeding up resistance detection – translation of molecular DST

Molecular techniques can significantly reduce time to DST, if the relationship between genotype, phenotype and clinical outcome is clarified. ReSeqTB is a global initiative to standardize WGS data analysis and to facilitate the analysis of results. The CRyPTIC (Comprehensive Resistance Prediction for Tuberculosis) consortium plans to provide MIC determination of 14 common TB-drugs as well as WGS data for more than 30 000 clinical *M.tuberculosis* isolates (188). The aim is to uncover silent and resistance-associated mutations and develop an algorithm allowing the identification of resistant as well as susceptible isolates (67). Our understanding of the impact on clinical outcome needs to be improved. Future developments involve WGS directly from sputa, dealing with contamination of human DNA as well as increased accessibility of the technique. Although unlikely to replace phenotypic DST globally in the near future, an improved WGS could be used to more rapidly guide the initial choice of therapy and for a more individualised treatment regimen.

Dried blood spot in clinical practice

To further facilitate TDM in clinical practice, dried blood spot (DBS) as a bio-sampling method has been developed for many TB-drugs, such as rifampicin (189) and moxifloxacin (190). Figure 16 shows DBS sampling with a simple finger-prick, enabling TDM even in remote areas, since the DBS card can be stored and transported in room temperature. The methodology to analyse DBS should include all main second-line drugs, including bedaquiline.

Figure 16. Finger-prick for dried blood spot sampling.

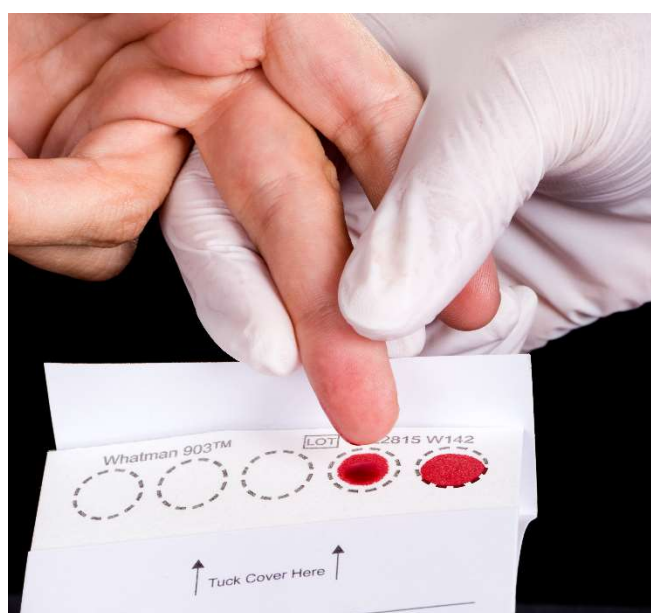


Photo: Anders Norderman, Medical Image, Karolinska University Hospital Solna

Model-based TDM for dosage guidance

Traditionally, a blood sample is collected after two weeks' treatment, for the drug to be at steady state and to account for autoinduction. With model-based TDM, a few blood samples can be collected and analysed already at treatment start, together with information of time of drug intake and blood sampling, age, gender and creatinine clearance. The model can then predict the optimal dose for that individual, taking autoinduction into account. The dose might be further revised when the result of the individual MIC is available, considering MIC variability. A model-based TDM approach has been developed for rifampicin by the Department of Pharmaceutical Biosciences, Uppsala. Such a tool would enable optimized dosages earlier on in treatment. Potential improvement of the model involves the inclusion of disease severity, immune status, comorbidities, pharmacogenomics and to develop models for groups needing extra attention, such as children and pregnant women.

There are several promising studies regarding treatment optimization of susceptible and MDR-TB (86). There are also a number of TB-specific drugs under investigation (www.newtbdrugs.org). Reliable biomarkers are needed to simplify TB trials and improve clinical care. This is especially needed regarding treatment length, since patients might require different durations of treatment, depending on disease severity and comorbidities. A biomarker to guide individualised duration of treatment, as well as a marker predicting risk of latent TB infection progressing to active TB, would be ideal.

Finally, TB deserves attention as the threat to public health and the global emergency it is. Regrettably, *M.tuberculosis* was not even included in the WHO's list of bacteria in 2017 for which new drugs are urgently needed (191). This omission is indefensible; TB should receive the utmost attention in governance and policy making and we have to hope that the high-level UN-meeting in September 2018 has a substantial impact.

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